EXPERIMENTAL AND COMPUTATIONAL STUDIES ON LIPOPROTEIN LIPOLYSIS AT ACIDIC pH

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ACADEMIC DISSERTATION

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To my loved ones

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-III:

I. **Lähdesmäki K**, Plihtari R, Soininen P, Hurt-Camejo E, Ala-Korpela M, Öörni K, Kovanen PT. Phospholipase A_2-modified LDL particles retain the generated hydrolytic products and are more atherogenic at acidic pH. *Atherosclerosis* 207(2):352-359, 2009.

II. **Lähdesmäki K**, Ollila OHS, Koivuniemi A, Kovanen PT, Hyvönen MT. Membrane simulations mimicking acidic pH reveal increased thickness and negative curvature in a bilayer consisting of lysophosphatidylcholines and free fatty acids. *Biochimica et Biophysica Acta (BBA) – Biomembranes* 1798(5):938-946, 2010.

III. **Lähdesmäki K**, Öörni K, Jauhiainen M, Hurt-Camejo E, Kovanen PT, Acidity and lipolysis by secretory phospholipase A_2 group V strongly increase the binding of apoB-100-containing lipoproteins to human aortic proteoglycans. *Submitted for publication.*

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ABBREVIATIONS

ACAT	acyl coenzyme A:cholesterol acyltransferase
Apo	apolipoprotein
ApoB-100	apolipoprotein B-100
ApoC-III	apolipoprotein C-III
ApoE	apolipoprotein E
BSA	bovine serum albumin
CE	cholesteryl esters
DIT	diffuse intimal thickening
FFA	free fatty acid
FID	free induction decay
GAG	glycosaminoglycan
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDL	high-density lipoprotein
HSA	human serum albumin
HPLC	high pressure liquid chromatography
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LDLR	LDL receptor
Lp-PLA_2	lipoprotein associated PLA_2
LRP-1	LDL receptor-related protein 1 receptor
lysoPC	lysophosphatidylcholine
M-CSF	macrophage colony-stimulating factor
MD	molecular dynamics
NAD^+	nicotinamide adenine dinucleotide
NEFA	non-esterified fatty acid
NMR	nuclear magnetic resonance
ox-FFA	oxidized free fatty acid
ox-LDL	oxidized LDL
ox-PC	oxidized phosphatidylcholine
PAF	platelet-activating factor
PAF-AH	platelet-activating factor acetylhydrolase
PC	phosphatidylcholine
PG	proteoglycan
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
$sPLA_2$	secretory phospholipase A_2
$sPLA_2$-IIA	secretory phospholipase A_2 group IIA
$sPLA_2$-III	secretory phospholipase A_2 group III
$sPLA_2$-V	secretory phospholipase A_2 group V
$sPLA_2$-X	secretory phospholipase A_2 group X
sVLDL	small very-low-density lipoprotein
SM	sphingomyelin
SMC	smooth muscle cell
SR	scavenger receptor
TLC	thin layer chromatography

ABSTRACT

Atherosclerosis is a disease of the arteries; its characteristic features include chronic inflammation, extra- and intracellular lipid accumulation, extracellular matrix remodeling, and an increase in extracellular matrix volume. The underlying mechanisms in the pathogenesis of advanced atherosclerotic plaques, that involve local acidity of the extracellular fluid, are still incompletely understood.

In this thesis project, my co-workers and I studied the different mechanisms by which local extracellular acidity could promote accumulation of the atherogenic apolipoprotein B-100 (apoB-100)-containing plasma lipoprotein particles in the inner layer of the arterial wall, the intima. We found that lipolysis of atherogenic apoB-100-containing plasma lipoprotein particles (LDL, IDL, and sVLDL) by the secretory phospholipase A_2 group V ($sPLA_2$-V) enzyme, was increased at acidic pH. Also, the binding of apoB-100-containing plasma lipoprotein particles to human aortic proteoglycans was dramatically enhanced at acidic pH. Additionally, lipolysis by $sPLA_2$-V enzyme further increased this binding. Using proteoglycan-affinity chromatography, we found that sVLDL lipoprotein particles consist of populations, differing in their affinities toward proteoglycans. These populations also contained different amounts of apolipoprotein E (apoE) and apolipoprotein C-III (apoC-III); the amounts of apoC-III and apoE per particle were highest in the population with the lowest affinity toward proteoglycans.

Since PLA_2-modification of LDL particles has been shown to change their aggregation behavior, we also studied the effect of acidic pH on the monolayer structure covering lipoprotein particles after PLA_2-induced hydrolysis. Using molecular dynamics simulations, we found that, in acidity, the monolayer is more tightly packed laterally; moreover, its spontaneous curvature is negative, suggesting that acidity may promote lipoprotein particles fusion. In addition to extracellular lipid accumulation, the apoB-100-containing plasma lipoprotein particles can be taken up by inflammatory cells, namely macrophages. Using radiolabeled lipoprotein particles and cell cultures, we showed that $sPLA_2$-V–modification of LDL, IDL, and sVLDL lipoproteins particles, at neutral or acidic pH, increased their uptake by human monocyte-derived macrophages.

I. INTRODUCTION

Atherosclerosis develops slowly over decades due to both genetic and environmental factors (Lusis 2000). Early and intermediate atherosclerotic lesions are clinically silent, but some of them progress into advanced lesions prone to rupture that can cause disability or death. The first symptom of atherosclerosis often occurs as a sudden, potentially life-threatening cardiovascular event, and therefore research on the underlying mechanisms promoting atherosclerotic lesion progression is important. Complications of atherosclerosis are the most common cause of death in the Western world and the prevalence of the disease is increasing worldwide (Murray 1997, Lloyd-Jones 2009).

Apolipoprotein B-100-containing lipoprotein particles transport lipids in blood; in atherosclerosis, these lipoprotein particles accumulate in the inner layer of the arterial wall, the intima (Williams 1995, Williams 1998). A fraction of the circulating lipoprotein particles enters the intima through the endothelium in transcytotic vesicles, and in the intima they bind to the extracellular matrix components, mainly to arterial proteoglycans (Wight 2004). The matrix-bound lipoprotein particles can become oxidized in reactions catalyzed by metal ions or by enzymes, and become lipolyzed and proteolyzed by various enzymes secreted by intimal cells. Recently, in the mouse, secretory phospholipase A_2 group V (sPLA$_2$-V) enzyme was shown to promote atherosclerotic lesion progression, and a Western-type high fat diet was shown to increase the secretion of this enzyme in the atherosclerotic lesions (Boström 2007, Rosengren 2006b). Further evidence is emerging on the protective role of phospholipase A_2 (PLA$_2$) inhibitors in the treatment of atherosclerosis in humans, and there are several ongoing clinical trials on novel inhibitors of PLA$_2$ enzymes (Karakas 2009, Riley 2009, Rosenson 2009b).

Lipoprotein particles bound to proteoglycans are more sensitive to a number of modifications (Wight 2004) and modified lipoprotein particles are prone to ingestion by inflammatory cells called macrophages. Uptake of modified lipoprotein particles by macrophages can result in the formation of highly oxygen-consuming foam cells loaded with lipid droplets, which sets off a cascade of inflammatory processes and leads to further lipid retention (Libby 2002). Modified lipoprotein particles can also aggregate and fuse to form extracellular lipid droplets, which may be ingested by macrophages. Importantly, the intimal layer thickens due to intra- and extracellular lipid and

extracellular matrix accumulation. The intimal thickness may reach 1500 ± 350 µm (Sluimer 2008), which greatly exceeds the maximal oxygen diffusion distance of 100-200 µm in tissues (Torres Filho 1994). This results in insufficient supply of oxygen to cells in intima. The combination of increased oxygen demand by highly oxygen-consuming activated cells and low oxygen supply leads to hypoxia in macrophage-rich areas (Sluimer 2009, Hultén 2009). Under hypoxia, cells switch to anaerobic metabolism and thus secrete metabolites acidifying the local extracellular milieu (Leppänen 2006, Naghavi 2002).

The purpose of the present thesis is to examine the influence of acidity on intra- and extracellular $sPLA_2$-V-promoted atherogenic processes. In particular, this study investigates the role of $sPLA_2$-V enzyme in the following processes: (1) lipolysis of lipoprotein particles, (2) acidity-induced changes in the surface monolayer of lipoprotein particles, (3) binding of lipoprotein particles to human aortic proteoglycans, and (4) uptake of lipoprotein particles by human monocyte-derived macrophages.

II. LITERATURE REVIEW

1. PATHOGENESIS OF ATHEROSCLEROSIS

The arterial wall consists of three distinct morphological layers separated by elastic laminae: intima, media, and adventitia. Atherosclerosis develops in the intimal layer from early fatty streaks to advanced fibrous plaques via a slow process starting already at the fetal stage, and is characterized by lipid and extracellular matrix accumulation and intimal layer thickening (Stary 2000). Importantly, arteries normally have both thin and thick segments, and atherosclerosis preferentially develops in the thick atherosclerotic-prone areas located near bifurcations of arteries and at the openings of vessel branches where arteries have adjusted their intimal thickness to equal flow at all points (Stary 2000). The intima is morphologically divided into two layers: a proteoglycan-rich layer (subjacent to the lumen) and an underlying musculoelastic layer (Fig. 1). The proteoglycan-rich intimal layer is composed of proteoglycans, collagens, and elastins, whereas the musculoelastic intimal layer is composed of smooth muscle cells (SMCs), and contains more elastins and collagens than the proteoglycan-rich intimal layer. The intima is separated from vascular lumen by a single layer of endothelial cells, and lipoproteins filtrate into the intima from blood through the endothelium in transcytotic vesicles (Steinberg 1985). In particular, transcytotic vesicles can carry lipoprotein particles having a diameter smaller than 75 nm (Nordestgaard 1988), i.e., low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and small very-low-density lipoprotein (sVLDL) particles.

Once lipoproteins have entered the intima, they may bind to the extracellular matrix and are, therein, prone to modification by various enzymes and oxidizing agents (Wight 2004). The extracellular matrix of the proteoglycan-rich intimal layer is mainly composed of arterial proteoglycans, and there is a highly specific interaction between proteoglycans and the apolipoprotein B-100 (apoB-100) moiety of lipoproteins (Iverius 1972, Wight 2004, Skålen 2002), which may be enhanced by lipoprotein modification (Öörni 2000). Modified lipoproteins are recognized by inflammatory cells and trigger the recruitment of more monocytes into the intima, where the monocytes differentiate into arterial macrophages that take up modified lipoprotein particles (Galkina 2009). Importantly, macrophages express, at their surface, scavenger receptors (SRs) (Goldstein 1979, Moore 2006) and also cell-surface proteoglycans

(Boyanovsky 2009a) that have a high affinity toward modified lipoprotein particles.

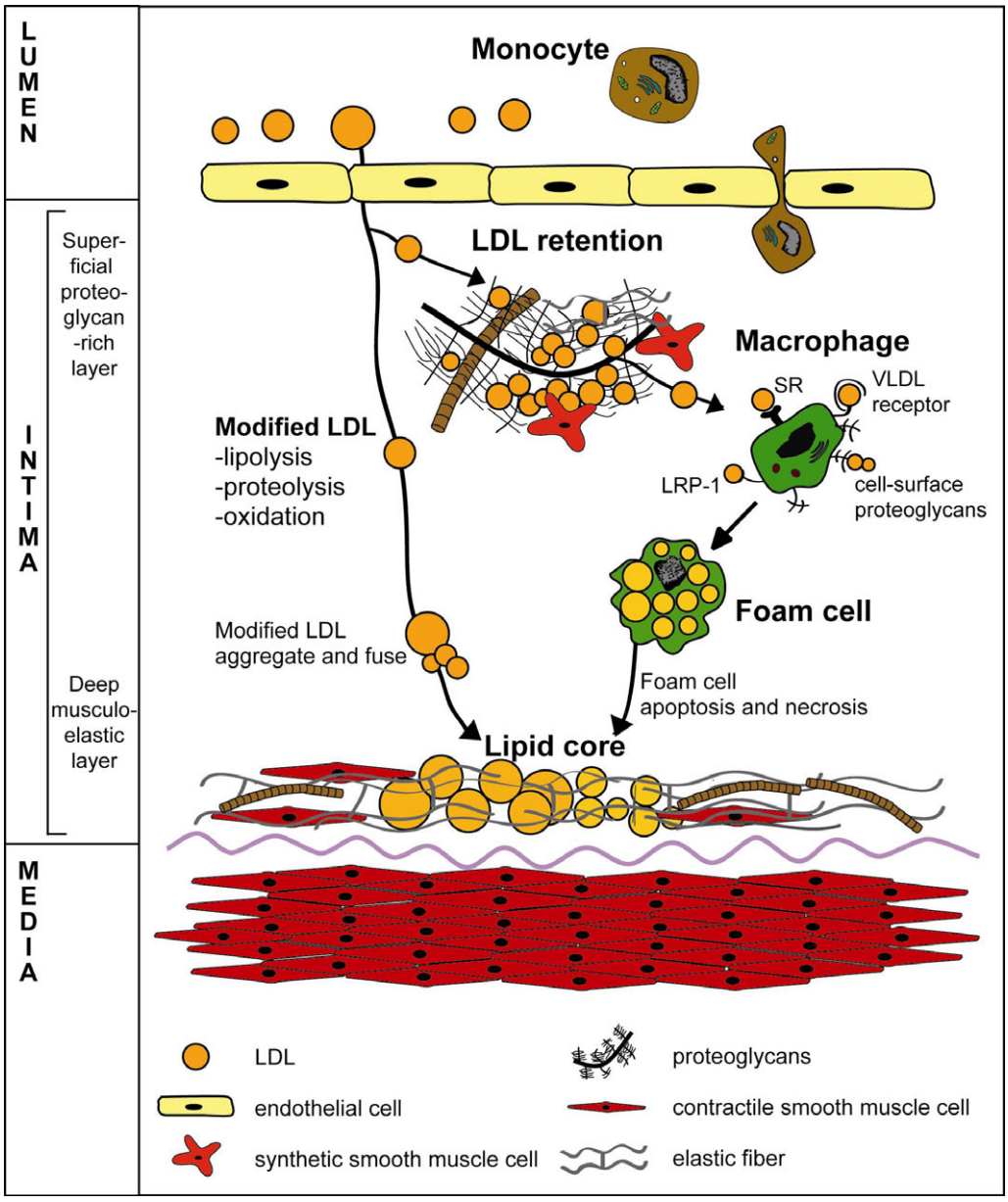

Figure 1. Accumulation of lipids in the atherosclerotic arterial intima.

Uptake of modified lipoproteins into macrophages may be beneficial during the initial stages of atherogenesis as it scavenges potentially inflammatory modified lipoproteins, preventing them from accumulating in the arterial wall. However, the uptake of modified lipoproteins is unregulated and leads to massive lipoprotein internalization, thus transforming macrophages into highly oxygen-consuming lipid-loaded foam cells (Goldstein 1979, Björnheden 1987). The foam cells attract more monocytes into the intima, stimulate SMC migration to the intimal layer from the media, and secrete pro-inflammatory cytokines and reactive oxygen species resulting in an amplification of the local

inflammatory response (Libby 2002). Thus, atherosclerotic intima resembles other chronic inflammatory sites in that it contains a large number of activated immune cells, such as monocyte-derived macrophages, mast cells, and T-cells (Libby 2002, Hansson 2005). The extracellular fluid in inflammatory tissues is known to be acidic (Lardner 2001). Thus, in analogy to other inflammatory and ischaemic sites, the atherosclerotic lesion areas, with clusters of inflammatory cells, have locally acidic extracellular pH (Naghavi 2002).

Foam cells may aggregate and form a fatty streak, which is the first visible sign of the oversupply of lipids to the arterial intima (Stary 2000). However, in the earliest stage of human coronary atherosclerosis, extracellular clusters of modified and fused apoB-100-containing lipoprotein particles form deposits within the extracellular matrix of the proteoglycan-rich layer (Pasquinelli 1989, Nakashima 2007). In time, increasing numbers of lipid droplets accumulate both in foam cells and within the extracellular matrix. The lipid deposits found in the core regions of fibrous plaques are almost entirely extracellular. The extracellular lipid droplets may be derived from either the necrosis of cells containing intracellular lipid droplets or from direct formation of lipid droplets in the extracellular space. The latter possibility is supported by: 1) the size and composition of the extracellular lipid droplets, both of which differ from those of intracellular lipid droplets (Guyton 1990), and 2) the observation that extracellular lipid deposition may form independent of foam cell death (Nakashima 2007).

During the progression of atherosclerotic lesions, the thickness of intima increases due to lipid accumulation, and, more importantly, due to increased deposition of the extracellular matrix components. Thus, the extracellular matrix is remodeled during lesions progression. SMCs play a key role in this remodeling by migrating from the media to the intima, where they proliferate and secrete extracellular matrix components (Doran 2008). Increased intimal thickness, which is beyond the oxygen diffusion distance, with the increased oxygen demand caused by activated macrophages, may lead to hypoxic conditions. Indeed, regions of severe hypoxia have been detected in advanced lesions, particularly at the junction of the proteoglycan and musculoelastic layers, where macrophage foam cells typically accumulate (Björnheden 1999, Sluimer 2008).

Initially, no capillaries are found in the intimal layer; however hypoxia promotes angiogenesis, a process in which neovascular sprouts originating

from the adventitial vasa vasorum enter the lesion (Sluimer 2008). These new microvessels provide oxygen and nutrients, helping to restore the ATP levels in cells within lesions. Angiogenesis is also promoted by various growth factors, chemokines, cytokines, and proteinases present in the lesions (Fraisl 2009). However, neovascularization may be maladaptive and may be a pathophysiological consequence of lesion progression because the inflammatory cells may induce rupture of the fragile neovessels, thereby causing intraplaque hemorrhage and ensuing plaque destabilization (Ribatti 2008, Sluimer 2009). Additionally, hypoxia plays a key role in many other atherogenic processes by also promoting lipid accumulation, inflammation, and depletion of ATP (Hultén 2009). Of particular interest are the observations of both hypoxia and local acidity in advanced atherosclerotic lesions (Björnheden 1999, Sluimer 2008, Naghavi 2002).

2. EXTRACELLULAR ACIDIFICATION OF THE INTIMA

Local areas with low pH have been measured in deeper layers of atherosclerotic plaques (Naghavi 2002). Extracellular pH of endarterectomized human carotid artery plaques were measured by the following two complementary methods: pH electrodes and visualization with pH-sensitive fluorescent dyes. Measurements with the electrode could reach superficial layers at 200 µm, and showed acidic pH values of 6.8. With the fluorescent dyes, tissue sections covering the entire plaque could be visualized, and low pH areas were observed in the deep layers of the atherosclerotic plaque samples. Many lines of evidence provide support for low pH in the atherosclerotic arterial intima and are discussed in more detail in the following chapters.

2.1 Intimal thickening

Based on morphological studies on human autopsy specimens, before atherosclerosis had developed, the atherosclerosis prone areas in the coronary arteries, and in the abdominal aorta, have diffuse intimal thickenings (DIT) reaching ~250 µm (Stary 1992, Nakashima 2002, Nakashima 2008). SMCs are the major source of extracellular matrix proteoglycans in the deep layer of DIT, where atherogenic lipids deposit eccentrically. Following the enrichment of proteoglycans and lipid deposition, immune cells infiltrate these regions.

Extracellular matrix remodeling, together with intra- and extracellular lipid accumulations, thicken the intima larger than DIT present in human arteries before atherosclerotic lesions develop. Plaque formation, involving increasal in

extracellular matrix and lipid-rich deposits, leads to a dramatic increase in the intimal thickness, e.g., in carotid plaques, thicknesses of 1500 ± 350 µm have been measured (Sluimer 2008) that, in turn, may promote extracellular acidification via several mechanisms.

2.2 Hypoxia

Because the intimal layer is avascular, the intimal cells depend on diffusion for nutrition and oxygen supply. Normally, cells in tissues are within 20-30 µm from a capillary, but in thickened atherosclerotic plaques, the cells are pushed much further from the capillaries. Thus, since the maximal oxygen diffusion distance in tissues is ~100-300 µm (Torres Filho 1994), the intimal thickness of, for example advanced carotid plaques, significantly exceeds the oxygen diffusion limit (Sluimer 2008). Therefore, the oxygen tension in pathological tissues is reduced (hypoxia) to values below 10 mmHg (<1 %), which is significantly less compared to healthy tissues, where the oxygen tension is 20-70 mmHg (2.5-9.0 %) (Lewis 1999). Also, local oxygen consumption in the atherosclerotic intima is increased due to the increasing number of recruited monocyte-derived macrophages that transform into highly oxygen-consuming lipid-loaded foam cells (Murdoch 2005, Björnheden 1987). Hence, the local decreased oxygen supply, together with increased oxygen consumption of activated cells, leads to hypoxic zones in advanced atherosclerotic lesions (Björnheden 1999, Sluimer 2008). Indeed, low oxygen concentrations are found in atherosclerotic plaques (>500 µm) at depths exceeding the oxygen diffusion distance; furthermore, in particular, macrophage-rich areas in advanced plaques have regions of severe hypoxia (Björnheden 1999, Sluimer 2008, Leppänen 2006). Additionally, despite their location well within the oxygen diffusion distance, some subluminal (20-30 µm) foam cells are already hypoxic (Sluimer 2008).

The hypoxic conditions of advanced atherosclerotic lesions play a role in the development of atherosclerosis by several mechanisms (Hultén 2009). By reducing the β-oxidation of fatty acids and increasing the triglyceride and fatty acid biosynthesis, hypoxia increases the accumulation of triglyceride-containing intracellular lipid droplets in macrophages (Boström 2006). Hypoxia also promotes inflammation by enhancing macrophage-induced secretion of chemokines that recruit T-cells to plaques (Danielsson 2008). Furthermore, hypoxia may induce changes in the composition of extracellular matrix. For example, proteoglycans SMCs synthesize under hypoxia differ in

their glycosaminoglycan composition (Figueroa 1999). With regard to atherosclerotic plaque acidification, under hypoxic conditions macrophages switch to anaerobic glycolysis to regenerate ATP, which increases their proton release (Levin 2003).

2.3 Macrophage adaptation to hypoxia generates acidic metabolites

Intimal cells can adapt to low-oxygen and nutrient supply (Roiniotis 2009). This adaptation is seen in concentration gradients of energy metabolites across the arterial wall, measured with bioluminescence imaging (Levin 2003, Leppänen 2006). Under hypoxic conditions, the energy metabolites of the intimal cells, ATP, glucose, glycogen, and lactate, have heterogeneous intimal distributions. The majority of ATP production of macrophages under hypoxic conditions is created via anaerobic breakdown of glucose, which produces high local concentrations of lactate (>10mM), with a pKa value of 3.86. Potentially harmful accumulation of lactate is a good indirect marker for tissue hypoxia, and may partly explain the decrease in the extracellular intimal pH, because increased lactate production coincides with cellular acidosis (Toffaletti 1991).

The key role of oxygen is to maintain mitochondrial ATP production in cells. Hydrolysis of ATP molecule releases one proton, however, under normal conditions when the demand of ATP is met by mitochondrial respiration, the released protons are used by the mitochondria for oxidative phosphorylation and for maintaining the proton gradient in the intermembranous space (Robergs 2004). However, immune cells, particularly macrophages, efficiently adapt to hypoxia by relying on anaerobic glycolysis, which occurs in the cytosol (Levin 2003, Leppänen 2006). Oxygen independent glycolysis is more energy-inefficient than mitochondrial ATP production; it consumes 18 times more glucose per ATP molecule produced (McConnell 1992). To meet this high glucose demand, the HIF-1α-regulated glucose transporter GLUT-3 is upregulated in hypoxic macrophages (Sluimer 2008).

Figure 2 shows, schematically, the main reactions of the cytosolic anaerobic glycolysis that immune cells use to convert most of their glucose into lactate in order to regenerate ATP (Newsholme 1987). In addition to 2 NADH, the net reactions produce 2 protons and 2 ATP, when glucose is converted to 2 pyruvates, and 1 proton and 3 ATP when glycogen is converted to 2 pyruvates. However, NAD^+ must be generated in order to maintain glycolysis. Under anaerobic conditions, tissues regenerate NAD^+ in the cytosol in a lactate

dehydrogenase catalyzed reaction, which converts pyruvate to lactate and consumes 1 proton per molecule. Therefore, the reaction of lactate production functions as a buffer against cellular proton accumulation. There is no net production of protons when converting glucose to lactate, and a decrease of one proton occurs when converting glycogen to lactate. However, when anaerobic glycolysis in the cytosol is coupled with ATP hydrolysis, the net reactions of glycolysis and ATP hydrolysis together release protons that cause the cellular acidosis (Robergs 2004). Anaerobic glycolysis is by far the most acidifying process in terms of protons produced per turnover of an ATP molecule. Lactate produced in the cytosol is removed from the cell by a monocarboxylate transporter, which also symports protons. As a result, lactates and protons leave the cell stoichiometrically. The protons also may cross the plasma membrane by a Na^+-H^+ exchanger and by proton pumps.

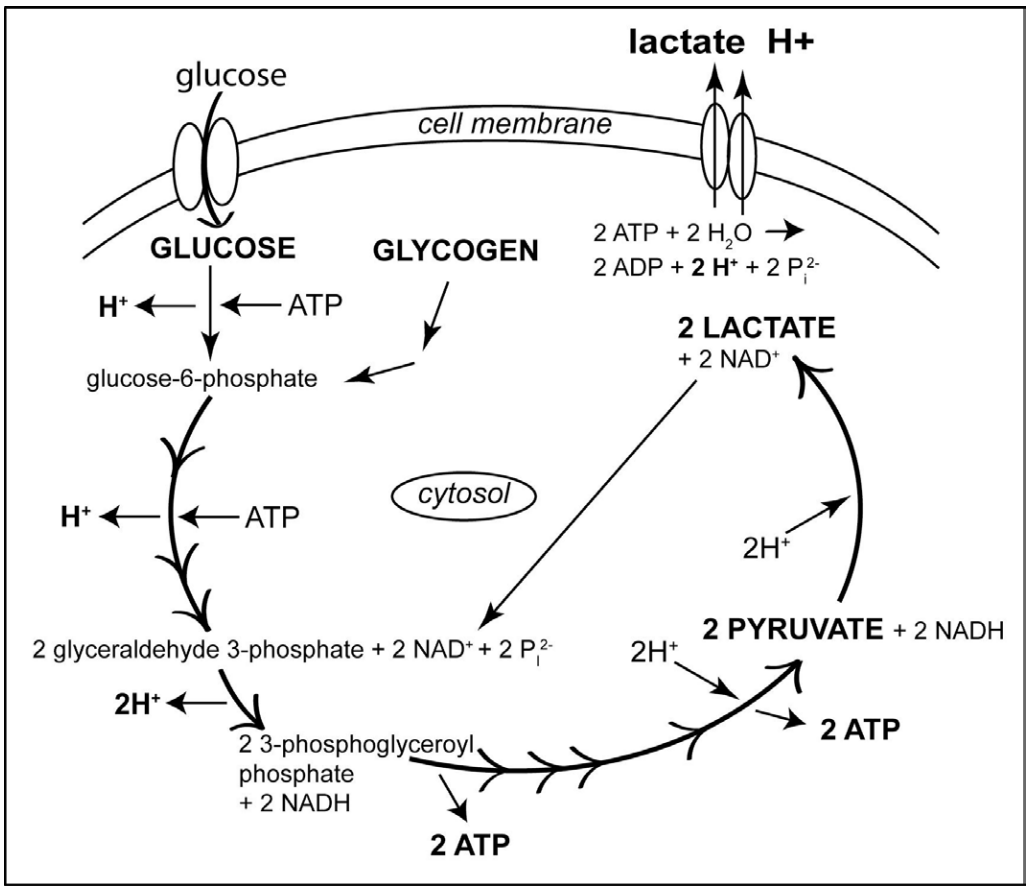

Figure 2. Schematic figure of the reactions of anaerobic glycolysis. For simplicity, only the reactions where ATP or protons are involved are shown. The product of anaerobic glycolysis, lactate, is secreted along with the protons produced in the hydrolysis of ATP.

Additionally, macrophages secrete matrix-destructive lysosomal enzymes with an acidic pH optimum (cysteine proteinases) together with protons, which may locally acidify the pericellular milieu (Punturieri 2000). The contact of

macrophages with LDL aggregates may also promote the formation of acidic extracellular compartments at the cell surface of macrophages (Haka 2009). The pH of the interstitial fluids of the atherosclerotic lesions will, of course, depend not only on the rate of proton release from the macrophages and other cells of the lesions, but also on the rate of proton removal from the lesions as well as the buffering capacity of the interstitial fluid (Leake 1997). With bioluminescence imaging in snap frozen arteries, the energy metabolites of arterial wall have been measured at high spatial resolution (Levin 2003). Thus, incubation at hypoxia resulted in increased lactate concentrations in the deep areas of the intima, whereas ATP, glucose, and glycogen concentrations were decreased close to zero. The ATP depletion observed may also result from the futile cycle of hydrolysis and re-esterification of cholesteryl esters that consumes ATP in lipid-loaded macrophages (Brown 1980).

2.4 Electrostatic interactions between proteoglycans and protons

Proteoglycans are large macromolecules that consist of polyanionic sugar chains called glycosaminoglycans (GAGs) covalently linked to a core protein. The polyanionic nature of GAGs enables electrostatic interactions between GAGs and cationic atoms or molecules or positively charged regions of macromolecules (Chakrabarti 1980). The net negative charges of GAGs are due to negatively charged carboxyl and sulfate groups. These groups are acids, the sulfate groups with pKa values below 2, and the carboxyl groups with pKa values between 3-5 (Chakrabarti 1980). Because of the electrostatic interactions between protons and negatively charged carboxyl and sulfate groups of GAGs, the proton concentration very near to GAG chains can be high, i.e. the pH is lower than the pH found further away (Maroudas 1988).

2.5 Acidic products generated by lipoprotein modifications

The reversible interactions of LDL with human arterial proteoglycans increases LDLs susceptibility to *in vitro* oxidation (Hurt-Camejo 1992). All the major cell types present in atherosclerotic lesions are able to oxidize LDL, i.e., endothelial cells (Henriksen 1981), SMCs (Henriksen 1983), macrophages (Parthasarathy 1986), and lymphocytes (Lamb 1992). Extensive LDL oxidation by these cells usually requires a catalyst. In the intima, iron or copper ions, nitric oxide or nitrite ions, myeloperoxidase, and lipoxygenase can catalyze the lipoprotein oxidation reactions. In plasma, iron and copper are carried in the proteins transferrin and caeruloplasmin. Decreasing pH increases LDL

oxidation by these proteins and also releases iron from transferrin enabling it to catalyze LDL oxidation (Lamb 1994a, Lamb 1994b). Decreasing pH also increases the reaction rate of LDL oxidation by iron or iron-cysteine in the presence or absence of cells (Morgan 1993, Morgan 1995). Interestingly, hypoxic macrophages oxidize LDL to a significantly higher extent than normoxic cells; they also express more of the LDL-oxidizing enzyme 15-lipoxygenase-2 (Rydberg 2004). Thus, LDL is oxidized within atherosclerotic lesions, and oxidized LDL (ox-LDL) is involved in several atherogenic processes (Berliner 1996, Navab 2004). It has been proposed that localized acidic pH found in advanced atherosclerotic lesions can be the underlying reason why atherosclerotic lesions are one of the few sites in the body where extensive LDL oxidation occurs (Leake 1997). LDL oxidation itself generates acids as end-products, conjugated dienes and hydroperoxides, which may further acidify the extracellular pH of the arterial intima (Leake 1997).

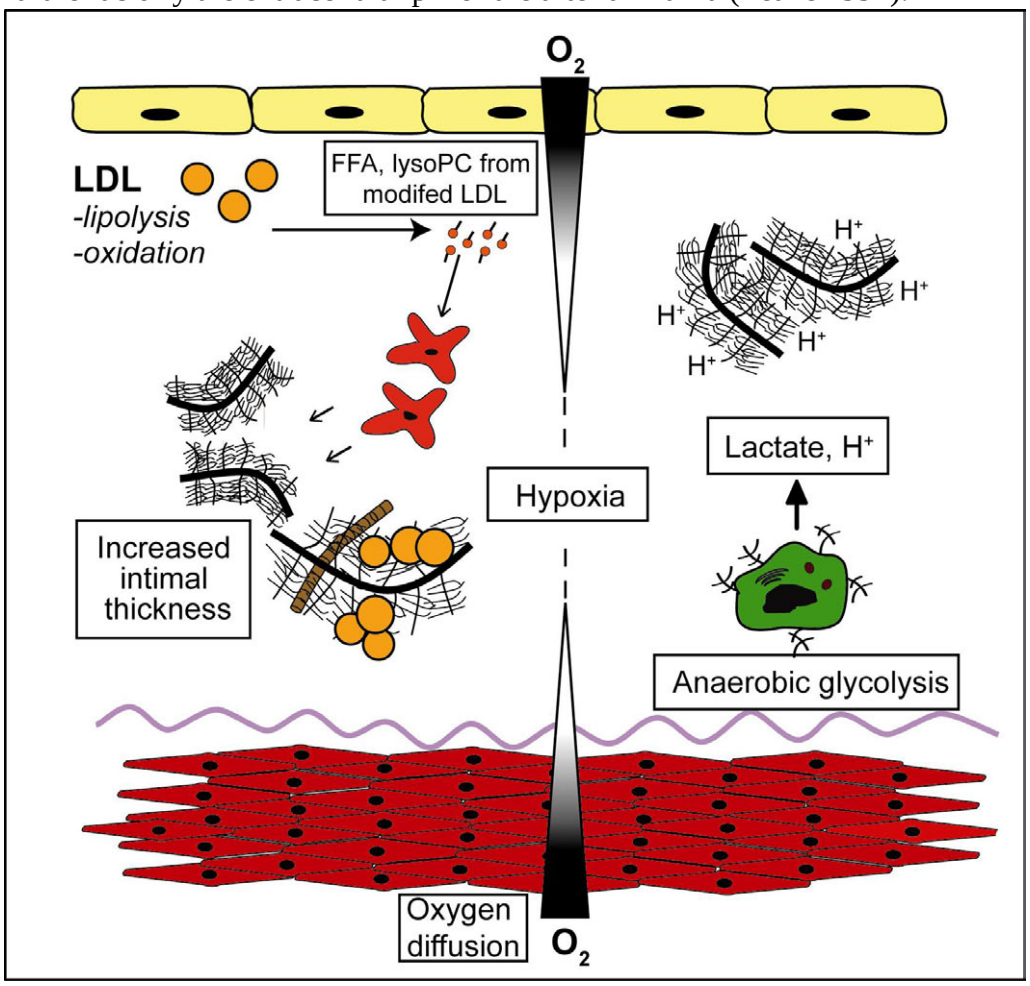

Figure 3. Acidification of the arterial intima.

Lipoprotein particles can also undergo phospholipolytic modifications in the arterial intima (Jönsson-Rylander 2008, Rosenson 2009a, Karabina 2010, Öörni 2009). Phosphatidylcholine (PC) is the main phospholipid component of

the monolayer covering lipoprotein particles. Its hydrolysis by the PLA_2 enzyme generates lysophosphatidylcholine (lysoPC) and free fatty acid (FFA) as end products. Also upon oxidation, the lysoPC content of LDL increases dramatically, compared to only 1–5 % lysoPC of the total PC content of native LDL (Chen 1997). This increase in lysoPC content can be the result of two sequential events: oxidation of the *sn*-2 chain of PC (ox-PC), followed by oxidized *sn*-2 fatty acid hydrolysis by lipoprotein-associated PLA_2 (Lp-PLA_2) or secretory phospholipase A_2 group X (sPLA_2-X), generating lysoPCs, and oxidized free fatty acids (ox-FFAs). Additionally, the acetyl moiety at the *sn*-2 position of platelet-activating factor (PAF) can be hydrolyzed, releasing acetate along with the lyso-platelet-activating factor (lysoPAF) (Stafforini 2009, Karabina 2010). Thus, sPLA_2 enzymes may hydrolyze PCs or ox-PCs (van Kuijk 1987, Matsumoto 2007), and release a wide spectrum of acidifying products to the intima, including hydroperoxides, acetate, lysophospholipids, FFAs, and ox-FFAs.

3. ATHEROGENIC APOB-100-CONTAINING LIPOPROTEIN PARTICLES

Lipoprotein particles are divided into classes differing in their density. This classification is primarily determined by protein and triglyceride contents where the less dense particles have a high triglyceride content and the more dense particles have a high protein content (Hilbert 2007). Cholesterol in plasma is carried by several lipoprotein particle classes that transport dietary and endogenously produced lipids. The four lipoprotein classes that carry endogenously produced lipids are: high-density lipoprotein (HDL), LDL, IDL, and VLDL, in the order of increasing particle diameter and triglyceride content (see Table 1.). In the postprandial state, dietary lipids are carried in chylomicrons and chylomicron remnants.

The apoB-100-containing lipoprotein particles are secreted into the circulatory system from the liver as large triglyceride-rich VLDL particles, which transport fatty acids to adipose tissue and muscle. In the circulation, VLDL particles are transformed, through the action of lipases and plasma lipid transfer proteins, into smaller cholesteryl ester-rich LDL particles. LDL particles are the principal plasma cholesterol carriers and serve as a source of cholesterol for most tissues of the body through receptor-mediated recognition of apoB-100 (Brown 1986). As a consequence of intravascular VLDL metabolism, the circulating apoB-100-containing lipoprotein particles are

heterogeneous in size, density, surface charge, lipid, and apolipoprotein compositions.

3.1 Lipoprotein particle structure

The structure of lipoprotein particles enables the water-solubility and transport of insoluble lipids in blood. Hydrophobic triglycerides and cholesteryl esters are located in the particle core and are solubilized by a surface monolayer of phospholipids, sphingolipids, and cholesterol. These particles are stabilized by proteins (Prassl 2009). Importantly, the so called apoB-100-containing lipoproteins, i.e. VLDL, IDL, and LDL, are considered atherogenic, and each particle contains a single molecule of non-exchangeable apoB-100 located at the particle surface. ApoB-100 is one of the largest single polypeptide chains known, containing 4536 amino acids (MW=513 000) (Yang 1986, Knott 1986). It is synthesized by the liver and together with apoE secreted on VLDL particles. After triglyceride removal in peripheral tissues, the remaining VLDL remnants are metabolized to LDL particles by further removal of the core triglycerides and the dissociation of apolipoproteins, other than the apoB-100. Hence, hydrolysis of VLDL results in IDL particles, and finally LDL particles.

Due to the size and hydrophobic nature of apoB-100, its structure, when associated with lipoprotein particles, is not well characterized (Johs 2006, Segrest 2001, Prassl 2009). ApoB-100 is located at the surface of lipoprotein particles, and its amphipathic α-helices and β-sheets represent the major lipid-associating motifs. The β-sheets are believed to penetrate more deeply into the particles and directly contact the neutral lipids of the core, playing a role in the organization of core lipids. Interestingly, the surface pressure of a lipoprotein particle decreases with decreasing particle size, and, with decreasing lipoprotein particle size, an increasing number of amphipathic helices of apoB-100 are associated with the surface lipids that modulate these surface pressure decreases (Segrest 2001).

In addition to apoB-100, lipoprotein particles also contain other smaller apolipoproteins that are divided into the following alphabetical classes: apo-A, B, C, D, E, etc. These are attached to lipoproteins through amphipathic helices that intercalate into the phospholipid monolayer and have various functions, including lipid transport in blood, enzyme regulation involved in lipid metabolism, and lipoprotein binding to receptors. The membrane association of

these small apoliproteins is sensitive to the surface pressure of the particles (McNamara 1996).

Table 1. Composition of the lipoproteins in human plasma*

		HDL	***LDL***	***IDL***	***VLDL***	***Chylo-microns***
	Particle size (nm)	*5 –12*	*18 – 30*	*25 – 35*	*30 – 80*	*75 – 1200*
	Density (g/ml)	*1.063 – 1.210*	*1.019 – 1.063*	*1.006 – 1.019*	*0.95 – 1.006*	*< 0.95*
Core lipids	*Cholesteryl esters (% dry weight)*	*10 – 20*	*35 – 50*	*22*	*10 – 22*	*2 - 5*
	***Triglycerides** (% dry weight)*	***2 – 7***	***4 - 10***	***20 - 30***	***45 - 65***	***80 – 95***
Surface lipids and proteins	*Cholesterol (% dry weight)*	*3 - 5*	*6 - 10*	*7 – 10*	*4 - 10*	*1 – 3*
	Phospholipids (% dry weight)	*20 - 35*	*15 – 24*	*20*	*15 - 20*	*3 – 9*
	***Proteins** (% dry weight)*	***40 - 55***	***18 - 25***	***15 - 20***	***5 - 10***	***1 - 2***
	Major apolipoproteins	*A-I,II,IV; C-I,II,III; D; E*	*B-100*	*B-100; C-I,II,III; E*	*B-100; C-I,II,III;E*	*A-I,II,IV; B-48; C-I,II,III; E*

* Values are taken from: (Hilbert 2007)

3.2 Lipoprotein particles entering the intima

Size is the major restricting factor affecting lipoprotein particles' ability to enter the arterial intima. Chylomicrons, chylomicron remnants, and large VLDL particles are primarily excluded from the intima. The particles that are able to enter the arterial intima are <75 nm. These also include the small VLDL (sVLDL) particles (Nordestgaard 1995). The triglyceride-rich IDL and sVLDL particles are larger than LDL particles, and have a slower rate of efflux from the arteries than LDL-sized particles (Proctor 2004). The plasma concentrations of IDL and sVLDL increase in familial combined hyperlipidemia, type III hyperlipidemia, chronic renal failure, and non–insulin-dependent diabetes mellitus. In these conditions, the triglyceride-rich lipoprotein particles are considered at least as atherogenic as LDL particles (Nordestgaard 1995). Also, a study in rabbits has demonstrated that IDL cholesterol, and IDL plus sVLDL cholesterol, are a better predictor of the extent of atherosclerosis than is LDL cholesterol (Nordestgaard 1991). Furthermore, results from some epidemiological studies have shown that

elevated triglyceride-levels increase the risk of coronary heart disease independent of LDL cholesterol levels, and an elevated level of IDL cholesterol has been suggested to be an independent risk factor for the development of atherosclerosis (Stalenhoef 2008, Niemi 2009).

Once the lipoprotein particles have entered the arterial intima, they can interact with the components of the extracellular matrix. The entrapment of lipoprotein particles in the extracellular matrix prolongs their residence time in the intima and predisposes them to modifications by enzymes and agents secreted by intimal cells (Pentikäinen 1997). Using immunohistochemical stainings, enzymatically modified lipoprotein particles have also been detected in the arterial intima (Torzewski 1998), and the extracellular lipid droplets that have aggregated and fused, possess qualities suggesting that they are derived from modified plasma LDL particles (Öörni 2000). Thus, lipids derived from plasma lipoproteins accumulate in the intima in the form of lipid droplets. The extracellular lipid droplets may be derived from apoB-100-containing lipoprotein particles or from dying foam cells. Lipid analysis of extracellular perifibrous lipid droplets in the lipid core has revealed that their cholesterol is mainly esterified with linoleate, as is also found in the core of circulating apoB-100-containing particles, whereas the intracellular lipid droplets in foam cells contain cholesterol esterified with oleate (Deckelbaum 1977, Smith 1974). Furthermore, electron microscopic pictures of the rather small diameters (40-200 nm) of the extracellular lipid droplets support the notion that the extracellular lipid droplets originate directly from lipoprotein particles rather than from the cytoplasmic lipid droplets of foam cells, which have much larger lipid droplet diameters (300-6000 nm) (Chao 1990).

4. MODIFICATION OF LIPOPROTEINS BY PHOSPHOLIPASE A_2

PLA_2 enzymes have important roles in diverse physiological processes that involve the turnover of membrane phospholipids. These include the digestion of foreign agents, such as bacteria and viruses, and the release of arachidonic acid for eicosanoid synthesis. The superfamily of PLA_2 enzymes include secretory, cytosolic, calcium-independent, and lysosomal forms, as well as the platelet-activating factor acetylhydrolase (PAF-AH) (Burke 2009, Murakami 2002). All PLA_2 enzymes act on the *sn*-2 position of phospholipids to liberate FFAs and lysophospholipids. PLA_2 enzymes have been proposed to generate high levels of hydrolysis products at highly localized nanodomains of

membranes, thereby directly affecting local membrane curvature (Brown 2003). The localized enzymatic action of PLA_2 in a phospholipid membrane can be understood by its scooting mode in which the enzyme binds to a membrane and catalyzes the hydrolysis of even hundreds of phospholipids without leaving the membrane (Berg 2001, Jain 2006).

The secretory PLA_2 ($sPLA_2$) and Lp-PLA_2 families are of interest regarding the hydrolysis of PC, the major phospholipid of lipoprotein particles. These hydrolyze the *sn*-2 chains of PC and generate both lysoPCs and FFAs (Öörni 2009, Wilensky 2009). The PCs and phosphatidylethanolamines (PE) in lipoproteins may also contain an ether-linked *sn*-1 alkyl chain, and Lp-PLA_2 or $sPLA_2$ acting on these lipids produces lysoPE, lysoPAF and FFA (Snyder 2002). Prior to their lipolytic modification, lipoprotein particles may also become oxidized (Matsumoto 2007). In the oxidation of phospholipids, radicals attack the unsaturated fatty acids in LDL particles, notably the *sn*-2 chains of phospholipids, and this lipid peroxidation leads to formation of hydroperoxides and release of aldehyde fragments (malondialdehyde; MDA and 4-hydroxynonenal; 4-HNE). Indeed, circulating LDL, despite the potent antioxidant capacity of plasma, has been shown to contain oxidized phospholipids (Ishigaki 2009), and the oxidation of lipoprotein particles has been shown to play an important role in atherogenesis (Navab 2004, Ishigaki 2009).

4.1 Secretory phospholipase A_2

The family of $sPLA_2$ enzymes generally have high numbers of disulfide bonds (6-8 bridges), and low molecular masses (13-18 kDa), with the exception of the notably large size of $sPLA_2$-III (~55 kDa) (Lambeau 2008, Valentin 2000, Boyanovsky 2009b, Rosenson 2009a). The $sPLA_2$ enzymes differ in their substrate specificity with respect to the polar headgroup size and the acyl chain composition and type of linkage (Gesquiere 2002, Chen 1998, Winget 2006). An interesting property of $sPLA_2$ enzymes (except $sPLA_2$-III) is their interfacial activation (Jain 2006). The surface of the enzyme that contacts the phospholipid surface is called interfacial binding surface (i-face). The interaction between the i-face and the aggregate substrate may control access to the substrate. Indeed, when bound to the phospholipid monolayer or bilayer of the aggregate substrate, the observed rate of hydrolysis of $sPLA_2$ is more than 1000 times greater than with monodisperse phospholipids (Jain 2006). Thus, the ability of $sPLA_2$ to bind to the phospholipid membrane is crucial for its

activity. The binding of $sPLA_2$ to phospholipid membrane is sensitive to the phospholipid chain melting phase transition region, which depends on pH, ions, membrane potential, proteins, and changes in hydration (Kinnunen 1991). However, despite growing knowledge on interfacial enzymes, and especially $sPLA_2$, the biophysical steps involved in interfacial recognition and adsorption are still only partially understood (Winget 2006). Also the efflux propensity of phospholipid substrate is an important factor in the hydrolysis of phospholipid molecules by several PLA_2 enzymes (Haimi 2010).

4.2 Secretory phospholipase A_2 enzymes in atherosclerosis

A number of studies have shown that elevated activity and plasma concentrations of PLA_2 enzymes increase the risk of cardiovascular events (Koenig 2006, Brilakis 2005, Kugiyama 1999, Liu 2003, Kugiyama 2000, Mallat 2007, Koenig 2009). The activity of PLA_2 enzymes present in the blood plasma have been shown to be increased by acidity and plasma dilution (Costello 1990). Since the intimal fluids are an ultrafiltrate of blood plasma and the plasma-derived proteins contained in it are diluted (Smith 1990), it is conceivable that in the intimal fluids the activity of PLA_2 enzymes is increased.

In the arterial intima, out of the ten known mammalian $sPLA_2$ enzymes, seven have been shown to have various patterns of distribution and production at all stages of atherosclerotic lesion development (Kimura-Matsumoto 2008). Also, Lp-PLA_2 has been shown in advanced atherosclerotic plaques, associated with apoptotic macrophages (Häkkinen 1999, Kolodgie 2006). Furthermore, the compositional analysis of lipoproteins isolated from human atherosclerotic arteries show decreased PC content, supporting the role of PLA_2 enzymes in the modification of lipoprotein particles (Camejo 1985, Tailleux 1993). Currently, of the $sPLA_2$ family members, the groups $sPLA_2$-IIA, $sPLA_2$-III, $sPLA_2$-V, $sPLA_2$-X, and also the Lp-PLA_2, are regarded as promoters of lipid accumulation in the arterial intima (Menschikowski 1995, Sato 2008, Wooton-Kee 2004, Curfs 2008). These enzymes have different lipolytic activities and substrate specificities, and thus have been suggested to play different roles in the development of atherosclerosis (Rosengren 2006a, Öörni 2009, Jönsson-Rylander 2008).

Extensive PLA_2 hydrolysis of lipoprotein particles may increase their atherogenicity by several routes: provoking their aggregation and fusion (Hurt-Camejo 2001a, Öörni 2005), increasing their binding to extracellular matrix

proteoglycans (Sartipy 1999, Öörni 1998), and increasing their uptake by macrophages (Menschikowski 1995, Boyanovsky 2005).

4.3 Role of secretory phospholipase A_2 group V in atherosclerosis

Recently it was shown in mouse models that a hyperlipidemic high-fat diet upregulates the expression of $sPLA_2$-V in the aorta (Rosengren 2006b). Also, by immunohistochemical stainings, it was shown that $sPLA_2$-V is present in human and mouse atherosclerotic lesions, and that the enzyme is associated with SMCs and foam cells in lipid core areas of the atherosclerotic plaques (Wooton-Kee 2004, Rosengren 2006b, Kimura-Matsumoto 2008). Importantly, $sPLA_2$-V enzyme was shown to have a role in the development of atherosclerosis in LDL receptor-deficient mice (Boström 2007). Thus, the expression of $sPLA_2$-V in bone marrow-derived cells increased atherosclerotic lesion area by 2.7-fold, while deficiency of the enzyme reduced atherosclerotic lesion area by 36 %. The plasma $sPLA_2$-V activity of the mice did not change, which further supports the idea that increased lipid deposition in the mice overexpressing $sPLA_2$-V was attributable to the lipolytic activity of this enzyme within the arterial intima.

$sPLA_2$-V shows high enzymatic activity toward lipoprotein phospholipids in human plasma (Rosengren 2006b). Lipolysis of LDL by $sPLA_2$–V reduces the LDL particle size and increases particle density (Wooton-Kee 2004). The small dense LDL particles are considered more atherogenic since they exhibit enhanced binding to extracellular matrix and cell-surface proteoglycans than normal-sized LDL particles (Sartipy 1999, Hurt-Camejo 2001b). Modification of LDL by $sPLA_2$-V can also contribute to intracellular lipid accumulation and to the formation of macrophage foam cells (Wooton-Kee 2004, Boyanovsky 2005, Boyanovsky 2009a). Interestingly, nuclear factor κB (NFκB), a transcription factor and a key regulator of inflammation that has been implicated in atherosclerotic processes (de Winther 2005), is translocated to the nucleus after macrophages have been incubated with the lipolytic products released from $sPLA_2$-V-modified LDL particles (Boyanovsky 2010). Consequently, incubation of macrophages with $sPLA_2$-V-modified LDL increases the expression of NFκB target genes, such as pro-inflammatory cytokines TNF-α and IL-6 (Boyanovsky 2010).

4.4 Release of bioactive lysophospholipids and free fatty acids

Several lipolytic and proteolytic enzymes are highly induced in the atherosclerotic arterial intima and lipoprotein particles are prone to extensive modifications. However, it is currently not known which specific enzymes of the human arterial intima are actually responsible for turning the intimal lipoproteins into atherogenic particles. Some of the lipolytic enzymes potentially involved in such atherogenic conversion of lipoproteins include: phospholipases (Öörni 2009), cholesteryl ester hydrolases (Suriyaphol 2002), lipoprotein lipase (Mattsson 1993, Ichikawa 2005, Pentikäinen 2002), and lysosomal acid lipase (Hakala 2003). Simultaneous action of the various enzymes that hydrolyze cholesteryl esters and phospholipids may generate vast amounts of lipolytic products from lipoprotein particles. The lipolytic products formed by the action of PLA_2, lysophospholipids and free fatty acids, are highly bioactive molecules, and they play a role in the atherogenicity of the lipolytically modified lipoproteins (Hurt-Camejo 2001a, Öörni 2009, Matsumoto 2007, Schmitz 2009).

FFAs have effects on several cell-types found in the arterial intima. FFAs induce adhesion molecule expression and secretion of various cytokines in endothelial cells, and they increase cholesterol uptake and reduce cholesterol efflux from macrophages (Suriyaphol 2002, Oram 2004). Overexposure of arterial SMCs to FFAs also augments production of matrix proteoglycans with increased size and LDL shows an increased affinity for these altered proteoglycans (Olsson 1999, Rodriguez-Lee 2007). More severely, human vascular endothelial cells and human SMCs incubated with high FFA-plasma, or a mixture of FFAs, become apoptotic (Oram 2004, Artwohl 2004, Artwohl 2009). FFAs can also induce apoptosis in macrophages, but only when carried in lipoprotein particles (Chung 1995).

LysoPC has direct proinflammatory and atherogenic effects on almost all cell-types present in atherosclerotic lesions, including: SMCs, T-cells, monocytes, macrophages, platelets, endothelial cells, and neutrophils (Matsumoto 2007, Schmitz 2009). With regard to monocytes, lysoPC acts as a chemoattractant that plays a role in the recruitment of more monocytes into the arterial intima (Quinn 1988); it also stimulates interleukin-1 beta (IL-1β) production by monocytes (Liu-Wu 1998). Thus, in atherosclerosis, lysoPC acts as an atherogenic lipid mediator between modified lipoprotein particles and the arterial cells. The effects of lysoPC can be partly explained by its chemical

properties (reviewed by Matsumoto 2007), comprised of a long hydrophobic fatty acyl chain and a large hydrophilic headgroup. This amphipathic nature gives lysoPC surfactant- and detergent-like properties. At low concentrations, lysoPC exists as single molecules in solution, which can readily insert into the outer layer of the cell membrane, but do not appear to flip into the inner layer. After exceeding its critical micellar concentration in solution, lysoPC forms small micelles with detergent-like properties. The small micelles may fuse with cell membranes and disturb membrane conformation, and even lyse cells. Importantly, the apoptotic cell death is initiated by the secretion of lysoPC that plays a role as an "eat-me" signal, inducing the recruitment of phagocytes (Lauber 2003). The lysoPCs of the modified lipoprotein particles may also induce a similar effect. The accumulation of lysoPC in lipoprotein particles reflects either its increased production via PLA_2 enzyme activity or its decreased catabolism via enzymatic reacylation (Matsumoto 2007). Thus, scavenger receptor expression on the surface of macrophages is upregulated by lysoPC (Kita 2000), and ox-LDL particles containing high amounts of lysoPC are avidly internalized.

5. EXTRACELLULAR RETENTION OF LIPOPROTEIN PARTICLES

The key initiating event in early atherosclerosis is the subendothelial retention of cholesterol-rich, atherogenic lipoproteins, which, once retained, provoke a cascade of responses that lead to disease in a previously non-lesional artery. This so-called *response-to-retention* hypothesis is regarded as a central paradigm in our understanding of the pathogenesis of atherosclerosis (Williams 1995, Williams 1998, Tabas 2007).

As discussed earlier, atherosclerotic lesions tend to develop in specific areas of arteries, the DITs, suggesting that there are some local factors promoting atherogenesis in these atherosclerosis-prone zones (Nakashima 2008). It appears that the higher lipoprotein concentration in atherosclerosis-prone areas mainly depends on selective lipoprotein retention and degradation (Schwenke 1989). After entering the arterial intima from the circulatory system, lipoprotein particles may become entrapped within the dense extracellular matrix network of the intima, and bind to the matrix components, especially to proteoglycans (Skålen 2002, Wight 2004).

5.1 Extracellular matrix proteoglycans

Proteoglycans provide elasticity and volume to the arterial wall, and are mainly produced and secreted by SMCs (Olsson 1999). In the structure of proteoglycans, GAG chains are covalently attached to a core glycoprotein. The GAG chains are linear, highly polyanionic polymers of repeating disaccharides (Chakrabarti 1980). Because of their high anionic charges, they bind electrolytes and water in extracellular fluids and form a hydrated gel that mediates mechanical strength and support of tissues.

The relative amount, molecular size, and type of GAGs are tissue-specific, and the proteoglycans may be classified by their predominant GAGs. The proteoglycan-rich intimal layer of the arterial wall is mainly composed of proteoglycans known as versican, biglycan and decorin (see Figure 4.).

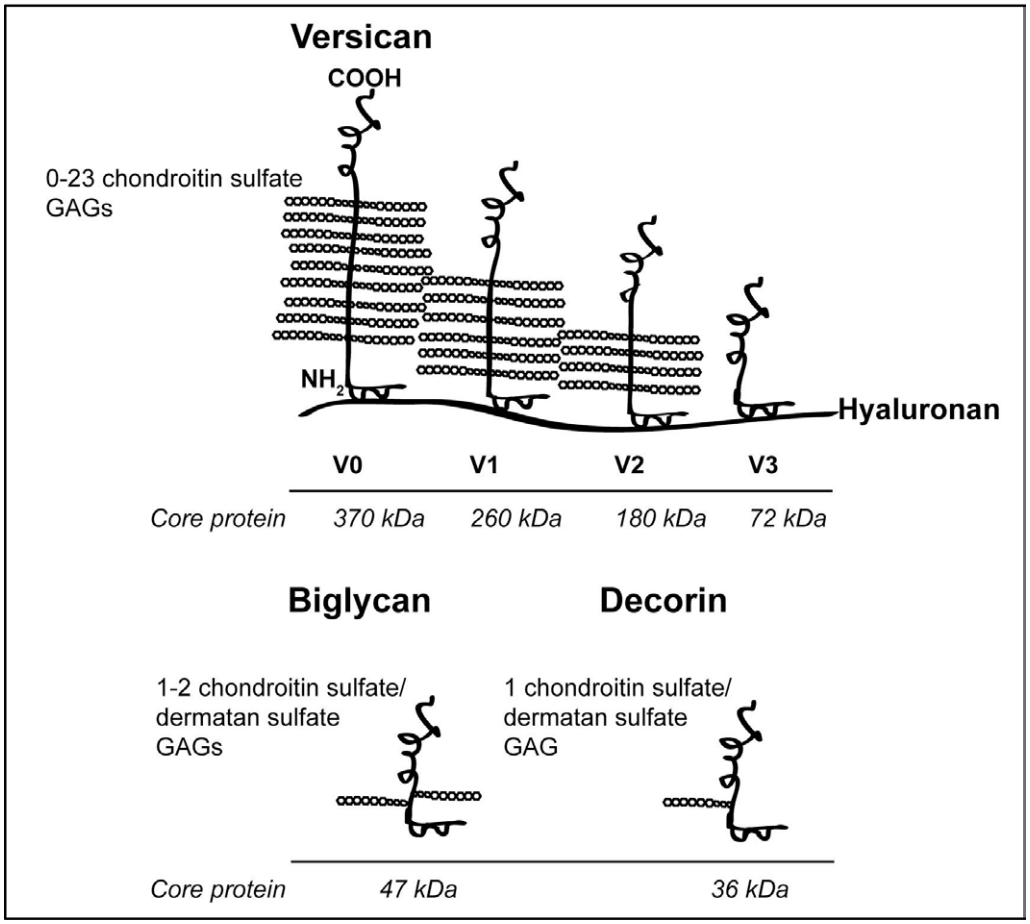

Figure 4. Structures of arterial wall proteoglycans versican, biglycan, and decorin.

Versicans have up to 23 chondroitin sulfate GAG chains attached to a core protein with four alternative sizes (V0-V3, 370-72 kDa) (Zimmermann 1989, Wight 2004). Also present are smaller proteoglycans known as biglycan and decorin, which have 1-2 chondroitin sulfate or dermatan sulfate GAG chains

attached to a ~40 kDa core protein (Cardoso 1994). Versican forms aggregates with hyaluronan, which is composed of a very long GAG chain. The hyaluronan-versican aggregates form enormous and tight proteoglycan networks within the extracellular matrix (Wight 2002).

Interestingly, proteoglycans in atherosclerosis-prone areas of the arteries have a higher binding affinity toward LDL than proteoglycans originating from low risk areas (Cardoso 1994). As the atherosclerotic lesions progress, the proteoglycan composition of the extracellular matrix changes; specifically, the chondroitin sulfate chains undergo structural changes that promote lipoprotein retention (Wagner 1986, Cardoso 1994).

The major changes to the extracellular matrix composition are made by SMCs, which migrate to the intimal layer from the media. After proliferation, SMCs secrete increased amounts of proteoglycans having a high affinity for LDL (Tao 1997, Doran 2008). Oxidation and hydrolysis of lipoprotein particles generates FFAs and lysoPCs, which play important roles in these processes, as discussed in section 4.4. LysoPC is one of the factors that increases the proliferation and migration of SMCs (Kohno 1998), and FFAs induce arterial SMCs to synthesize proteoglycans with increased affinity toward LDL (Olsson 1999, Rodriguez-Lee 2007).

5.2 Lipoprotein entrapment in the extracellular matrix

Lipoproteins directly bind to proteoglycans via electrostatic interactions between the negatively charged glycosaminoglycan side chains of proteoglycans and the positively charged residues, arginine and lysine, of apolipoproteins (Iverius 1972). In human apoB-100, site B (residues 3359-3369) is the functional proteoglycan-binding site (Borén 1998); however site A (residues 3148–3158) can also act co-operatively with site B. Site A is able to bind to proteoglycans after LDL particles have been lipolyzed by $sPLA_2$, which induces a conformational change in apoB-100 (Flood 2004). Indeed, $sPLA_2$-lipolysis increases lipoprotein binding to proteoglycans (Sartipy 1999, Öörni 1998). Interestingly, the association of $sPLA_2$-V enzyme with proteoglycans enhances its hydrolytic activity toward lipoproteins (Rosengren 2006b).

5.3 Lipoprotein aggregation and fusion

The outer monolayer of lipoprotein particles is mainly composed of phospholipids (Dowhan 2002), and a growing interest is the need to understand the biophysical properties of the monolayer lipids that may directly affect the

aggregation and fusion events of lipoprotein particles (Burger 2000), and the functions of various lipoprotein particle associated proteins (Phillips 2009). Indeed, lipids appear to play a key role in the fusion behavior of lipoprotein particles (Chernomordik 2008, Zellmer 1994, Knecht 2007). Fusion of lipoprotein particles requires strong membrane bending and highly curved non-bilayer lipid intermediates (Chernomordik 2008, Chernomordik 2003, Burger 2000), which are proposed to include phospholipids and lysophospholipids (Kooijman 2005, Kooijman 2003). The process of membrane bending depends on spontaneous curvature, which is influenced by external factors such as pH, temperature, and salt concentration. Both low pH and divalent cations can induce headgroup dehydration, and thus reduce the effective headgroup size by neutralizing the negatively charged headgroup regions. Reducing the headgroup size causes negative direction to monolayer curvature, which is an important parameter involved in membrane fusion (Chernomordik 2008). Additionally, the changes in atomic charges and molecular shapes of the membrane lipids (Kooijman 2003) may induce negative membrane curvature, and these may be altered *in vivo* by lipolytic enzymes or by oxidation.

Lipolytic, proteolytic, and oxidative modifications disrupt the surface of LDL particles and induce their fusion *in vitro*, which suggest that these modifications may also induce LDL fusion in the arterial intima. *In vitro*, proteolysis by α-chymotrypsin, trypsin, pronase, or cathepsins F, K, and S leads to fragmentation of apoB-100 and to fragment release from the LDL particle surface, and, importantly, the loss of protein fragments triggers the fusion of the particles (Piha 1995, Öörni 2004). Oxidation by copper-ions, and lipolysis by sphingomyelinase, produces lipid droplets of similar appearance to those found in the initial atherosclerotic lesions of the arterial intima (Pentikäinen 1996, Öörni 1998). Particle fusion may also occur when the lipoprotein particles are bound to the extracellular matrix of the arterial intima (Pentikäinen 1996). In fact, when bound to the human arterial proteoglycans, the rate of proteolytic LDL fusion is increased (Pentikäinen 1997). The PLA_2-modified lipoprotein particles have been shown only to aggregate (Öörni 1998, Wooton-Kee 2004), but they fuse when heparin-treated or heparin-bound (Hakala 1999).

6. INTRACELLULAR ACCUMULATION OF LIPOPROTEIN PARTICLES

Macrophages are the most abundant type of inflammatory cells in atherosclerotic lesions (~80% of lesional leukocytes), and they are present in lesions at all stages of atherosclerosis (Gerszten 2000). Macrophages are derived from blood monocytes, which, after circulating for several days, migrate into tissues where they differentiate into various tissue macrophages or dendritic cells. The outcome of the differentiation process into tissue macrophages depends on inherent properties of different monocyte populations as well as environmental factors in the tissue (Gordon 2005), leading to several macrophage phenotypes being present in atherosclerotic lesions (Johnson 2009, Mantovani 2009). There are two primary cytokines that promote monocyte differentiation into the macrophage phenotype: monocyte colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Barreda 2004). Macrophages differentiated with M-CSF are $CD68^{+}/CD14^{+}$, and represent the macrophage phenotype that predominates the diseased arterial intima (Waldo 2008). Already at the early stages of atherosclerosis, arterial macrophages take up lipoproteins by an unregulated mechanism, leading to intracellular lipid droplet accumulation (Stary 2000).

Macrophages have different kinds of cell-surface proteins and proteoglycans capable of binding and internalizing native, aggregated or modified lipoprotein particles. At least four major lipoprotein receptor types have been characterized that could be involved in arterial lipid uptake: 1) LDL receptor (LDLR), 2) LDL receptor-related protein 1 receptor (LRP-1), 3) VLDL receptor, and 4) scavenger receptors (SRs). Importantly, differentiated macrophages express LDLR poorly, but all the other receptor types are expressed abundantly in macrophages in atherosclerotic lesions, suggesting that no single receptor pathway is alone responsible for the uptake and degradation of lipoproteins by macrophages (Hiltunen 1998). Additionally, it has been suggested that arterial macrophages may have cell-surface receptors with an acidic pH-optimum for the binding of lipoprotein ligands (Ling 2004, Basu 1978). However, the function and characteristics of these cell-surface receptor proteins that have acidic pH-optimum are still poorly understood.

6.1 Receptor-mediated uptake

The uptake of native LDL particles to the cells is mainly by the LDLR, but differentiated macrophages express LDLRs poorly. LDLR binding to native LDL particles at neutral pH induces the formation of clathrin-coated endocytic vesicles. The vesicles quickly lose their clathrin-coating near the cell-surface and fuse with the endosome. During endosome maturation, acidic pH inside the vesicle induces the release of LDLR from its ligand. The receptor is then recycled to the cell-surface, whereas the late endosomes are fused to lysosomes (Brown 1986). LDLR is quickly downregulated in response to the entry of cholesterol into the cell, and so the LDLR pathway doesn't lead to excess intracellular cholesterol accumulation (Brown 1986). However, the LDLR has been proposed to be involved in the phagocytosis of large LDL self-aggregates, made *in vitro* by briefly vortexing LDL or by incubating LDL with phospholipase C enzyme (Khoo 1988, Suits 1989), and their uptake by the LDLR pathway may lead to formation of foam cells.

LRP-1, another receptor that belongs to the family of LDLRs (also known as α_2-macroglobulin receptor and CD 91). It is a very large (600kDa) multifunctional plasma membrane protein consisting of 4544 amino acids that structurally resembles four combined LDLR molecules (Herz 2001). LRP-1 is Ca^{2+}-dependent, like LDLR, and also has a neutral pH optimum for ligand binding (Moestrup 1990). The expression of LRP-1 is upregulated *in vivo* in both macrophages and SMCs in human atherosclerotic lesions (Lupu 1994). LRP-1 mediates, at least partially, the uptake of matrix-retained sphingomyelinase-aggregated LDL to macrophages in the presence of lipoprotein lipase (Sakr 2001). LRP-1 has also been shown to have a key role in the uptake of aggregated LDL by SMCs (Llorente-Cortes 2000, Llorente-Cortes 2002a), and LDL particles upregulate the expression of LRP-1 in SMCs (Llorente-Cortes 2002b).

The preponderance of modified LDL particles is taken up via a family of macrophage transmembrane proteins referred to as SRs (Goldstein 1979), which are divided to subclasses SR A-H (Moore 2006). SRs are able to bind bacterial pathogens, apoptotic cells, and modified endogenous proteins such as modified lipoprotein particles (Greaves 2009). The mature macrophages express SR-AI, SR-AII, SR-BI, and SR-BIII (also known as CD36), which are capable of binding modified LDL particles. SRs may be beneficial in the initial stages of atherosclerosis as they scavenge the modified lipoprotein particles,

but the unregulated nature of SRs activity that results in foam cell formation promotes atherosclerosis and chronic inflammation (Moore 2006). Oxidized phosphatidylcholines (ox-PCs) are one ligand that SRs recognizes on the surfaces of oxidatively damaged or apoptotic cells or ox-LDL (Hörkkö 2000, Boullier 2001). Macrophages recognize the ox-PCs and consequently take up the ox-LDL particles by SR-AI, SR-AII, and SR-BIII receptors (Podrez 2003, Suzuki 1997, Kunjathoor 2002). The binding of ox-LDL particles to SRs induces the formation of clathrin-coated endocytic vesicles, where ox-LDL particles are degraded in a way that is analogous to LDL in LDLR-induced endocytic vesicles (Brown 1986).

Another lipoprotein binding receptor expressed on the cell-surface of macrophages in atherosclerotic lesions is the VLDL receptor (Multhaupt 1996, Hiltunen 1998). This 846 amino acid peripheral remnant lipoprotein receptor belongs to the LDLR family (Takahashi 2004). It binds apoE-containing ligands but not LDL (Gianturco 1982, Van Lenten 1985, Kosaka 2001). Thus, the apoE-containing lipoprotein particles VLDL, IDL (VLDL remnants), chylomicron remnants, and Lp(a) bind to the VLDL receptor. The expression of the receptor and the internalization of ligands is unregulated, leading to the formation of foam cells (Takahashi 2004).

6.2 Receptor-independent uptake

Macrophages may also internalize lipoproteins through receptor-independent mechanisms. The activated human monocyte-derived macrophages, differentiated in the presence of human serum, internalize large amounts of native LDL through actin-dependent macropinocytosis of extracellular fluid (Kruth 2002, Kruth 2005). The same uptake mechanism is also observed, without cell activation, in macrophages differentiated in the presence of 10% fetal bovine serum containing M-CSF and IL-10 (Zhao 2006). Because the macropinocytotic uptake is not downregulated by the incoming cholesterol it leads to the formation of foam cells (Kruth 2002, Zhao 2006).

The uptake of $sPLA_2$-V-lipolyzed LDL particles also occurs through a receptor-independent mechanism that depends on cell-surface proteoglycans (Boyanovsky 2005, Boyanovsky 2009a). The role of syndecan-4 has been especially recognized in the uptake of $sPLA_2$-V-lipolyzed lipoprotein particles. Syndecans are cell-surface proteoglycans that contain glycosaminoglycan side chains composed of heparan sulfate and chondroitin sulfate (Wegrowski 2006).

Another, at least partly receptor-independent mechanism of LDL uptake is the phagocytosis of large LDL aggregates (Khoo 1988, Zhang 1997). Interestingly, PLA_2-lipolysis of LDL also promotes lipoprotein particle aggregation (Öörni 2005, Öörni 1998).

6.3 Intracellular lipid storage and degradation by macrophages

Normally, uptake of lipoproteins by cells results in the complete hydrolysis and degradation of their protein and lipid components by lysosomal enzymes having an acidic pH-optimum, such as cathepsins and lysosomal acid lipase found in the acidic environment of lysosomes. The lysosomal enzymes degrade apolipoproteins, and the degraded protein fragments are released into the cytoplasm and secreted from the cells (Brown 1986). The lysosomal enzymes also hydrolyze the cholesteryl esters of lipoprotein particles, and cholesterol is released into the cytoplasm. The cytoplasmic metabolically active pool of cholesterol is transported either to cell membranes, or directly to the endoplasmic reticulum. However, excessive uptake of lipoprotein particles can lead to accumulation of cholesterol in either the lysosomes or in different cellular membranes. In particular, in vascular cells, which are highly active in the uptake of ox-LDL, cholesterol accumulates in the lysosomes. The ox-LDL particles are poorly degraded in the lysosomes, possibly because of the resistance of ox-LDL to enzymatic hydrolysis, or because the reactive ox-LDL-associated moieties can inactivate lysosomal enzymes (Carr 2001).

The plasma membrane contains most of the total cellular cholesterol. However, if the cholesterol/phospholipid ratio of plasma membrane is too high, it can inhibit the function of some cholesterol efflux proteins as well as the function of other membrane proteins (Cantor 1999, Feng 2002). Excess cholesterol in the internal membranes may also interfere with integral membrane proteins, induce apoptosis, and initiate a pro-inflammatory response (Maxfield 2005). Therefore, intracellularly accumulating cholesterol is re-esterified in the cytoplasm by acyl-coenzyme A:cholesterol acyltransferase (ACAT), which esterifies cholesterol with fatty acids, preferably with oleate, and the formed cholesteryl esters are stored in cytosolic lipid droplets, which are also called lipid bodies (Brown 1986). If large amounts of lipoprotein particles are internalized into macrophages, the lipid droplets eventually occupy most of the cytoplasm, creating a foam cell.

The cholesteryl esters of intracellular lipid droplets are continually hydrolyzed by neutral cholesteryl ester hydrolase, which together with ACAT creates a futile cycle of hydrolysis and re-esterification that wastes ATP (Brown 1980). The lipid droplets also contain triglycerides, signifying that also fatty acids are stored as esters (Mattsson 1993, Boström 2006). The storage of triglycerides may result from the transmembrane translocation of fatty acids. This involves a number of membrane-associated fatty acid-binding proteins (FABPs) and albumin binding proteins that function as acceptors for fatty acids released from albumin or from lipoprotein particles (Glatz 1997). The direction of fatty acid migration through the plasma membrane is likely regulated by the transmembrane gradient of fatty acid concentration, and the fatty acids may diffuse across the phospholipid bilayer in protonated form (Pownall 2003). From the plasma membrane the fatty acids could be transported to the sites of oxidation or esterification (Stremmel 2001).

III. AIMS OF THE STUDY

Lipoprotein particles are known to undergo lipolytic modification, aggregate and fuse, bind to proteoglycans, and be taken up by monocyte-derived macrophages. These events are of importance in the selective lipid retention and accumulation within the intimal layer of the arterial wall. However, only a few studies address the effects of local extracellular acidity, present in the advanced atherosclerotic lesions, on the atherogenicity of the various apoB-100-containing lipoprotein classes. Therefore, the aim of the present study was to investigate whether acidic pH has an effect on the atherogenicity of lipoprotein particles that are able to enter the arterial intima.

The following specific questions were asked and answered:

1. Could acidic pH increase lipoprotein lipolysis by $sPLA_2$–V enzyme? If so, does the acidity influence the accumulation of highly bioactive lipolytic products in LDL particles?

2. What kinds of lipolysis-induced changes occur on the lipoprotein particle surface structure in acidic conditions? Will these changes promote lipoprotein particle aggregation and fusion?

3. Could lipolysis by $sPLA_2$–V enzyme, together with acidic pH, promote binding of the lipoprotein particles to arterial proteoglycans?

4. How do lipolysis by $sPLA_2$–V enzyme and acidic pH influence the uptake and degradation of lipoprotein particles by human monocyte-derived macrophages?

IV. MATERIALS AND METHODS

The different methods used in this thesis and their use in the three original publications are summarized in Table 2. These techniques have been described in more detail in the Methods sections of the original publications. If a more detailed description of the method has been published elsewhere, the reference is provided in Table 2.

Table 2. Methods used in the original publications I-III

Method	Used in publications	Reference
Binding and uptake of lipoproteins by macrophages	I, III	(Goldstein 1983)
Extraction and purification of aortic proteoglycans	I, III	(Hurt-Camejo 1990, Öörni 1997)
High pressure liquid chromatography	III	
^{3}H-labeling of lipoproteins	I, III	(Bolton 1973)
Isolation and culture of human monocytes	I, III	(Saren 1996)
Isolation of LDL, IDL, and sVLDL lipoproteins	I, III	(Havel 1955, Radding 1960, Lindgren 1972, Mahley 1979, Redgrave 1979)
Lipoprotein binding to proteoglycans	I, III	
MD-simulations	II	(Berendsen 1995, van Gunsteren 1996, Lindahl 2001)
Modification of lipoproteins	I, III	
NMR-spectroscopy	I	(Soininen 2005, Mierisová 2001, Soininen 2007)
Thin layer chromatography	I	

Lipoprotein isolation

Human VLDL (d < 1.006 g/ml), IDL (d = 1.006–1.019 g/ml), and LDL (d = 1.019–1.050 g/ml) were isolated from plasma (from the Finnish Red Cross), donated by healthy volunteers, using sequential ultracentrifugation in the presence of 3 mmol/L EDTA. Small VLDL (Sf 20–175) was isolated from the VLDL fraction by discontinuous density gradient ultracentrifugation. Isolated

lipoproteins were dialyzed against a solution containing 1mM EDTA and 150mM NaCl (pH 7.4).

^{3}H-labeling of lipoproteins

^{3}H-labeled lipoproteins, ^{3}H-LDL, ^{3}H-IDL, and ^{3}H-sVLDL were prepared by labeling the protein components according to the adapted Bolton-Hunter procedure, using the N-succinimidyl-^{3}H-propionate as a reagent. To obtain sPLA$_2$-V–modified ^{3}H-LDL, ^{3}H-IDL, and ^{3}H-sVLDL, ^{3}H-labeled lipoproteins were lipolyzed with sPLA$_2$–V as described below.

Modification of lipoproteins by phospholipolysis

Lipolytic modifications of the lipoproteins were performed using either bee venom PLA$_2$ or human recombinant sPLA$_2$-V enzymes. For lipolysis of LDL with bee venom PLA$_2$, the enzyme was first coupled to HiTrap NHS-activated Sepharose HP in a 1 ml column according to the manufacturer's instructions. LDL in PBS was added to the PLA$_2$-Sepharose and incubated at +37°C for 2 h. Sepharose-bound PLA$_2$ was removed by centrifugation and the supernatant containing PLA$_2$-modified LDL was collected. Lipolysis of LDL, IDL, and sVLDL lipoproteins with sPLA$_2$-V enzyme was performed by incubating the lipoproteins for 18h at +37°C with 100-130 ng/ml of sPLA$_2$-V in a buffer (20 mM HEPES/MES/PIPES, 5 mM CaCl$_2$, and 150 mM NaCl, 2% (w/v) BSA), at pH 7.5, 7.0, 6.5, 6.0 or 5.5. Lipolysis was terminated by the addition of EDTA or enzyme inhibitor compound 12e (6,7-benzoindole). After these phospholipolytic modifications, the degrees of lipoprotein phospholipolysis were determined with the NEFA-C-kit, which measures the amount of non-esterified free fatty acids in a sample.

Extraction and purification of aortic proteoglycans

Proteoglycans from the intima-media of human aortas were obtained at autopsy within 24 h of accidental death. The following is a brief summary of that process: proteoglycans were extracted from intima-media at 4°C for 24 h with 15 volumes of buffer containing 6 M urea and protease inhibitors. After extraction, the mixture was centrifuged at 100 000 g for 60 minutes. The supernatant was diluted with 6 M urea to give a final concentration of 0.25 M NaCl and loaded on a HiTrap Q column. The proteoglycans were eluted with a linear gradient of 0.25 M to 1.0 M NaCl, and the peaks at 280 nm were collected, dialyzed against water, and lyophilized. Glycosaminoglycans were quantified, and the amounts of proteoglycans were expressed in terms of their glycosaminoglycan content.

Lipoprotein binding to proteoglycans

Lipoprotein-proteoglycan interactions were investigated with proteoglycan-affinity chromatography and with proteoglycan coated microtiter well assays. To prepare human aortic proteoglycan-affinity column, human aortic proteoglycans (0.7 mg/ml) were coupled to a NHS-activated HiTrap column (5 ml), as described in the manufacturer's instructions. Native or $sPLA_2$-V–modified lipoproteins were injected (0.4 ml at 2.0 mg/ml) into the proteoglycan-column that had been equilibrated with buffer containing 10 mM HEPES for pH 7.5 or 10 mM MES for pH 5.5, 2 mM $CaCl_2$ and 2 mM $MgCl_2$. The material bound to the column was eluted with a gradient of NaCl. The elution of the samples was monitored by UV absorbance at 280 nm, and the accuracy of the gradient was monitored by measuring change in conductivity.

To measure lipoprotein binding to proteoglycans in a microtiter well assay, the wells of polystyrene 96-well plates were coated with human aortic proteoglycans (50 µg/ml in PBS) or BSA (5 mg/ml) by overnight incubation at +4 °C. The proteoglycan-coated wells were measured to contain about 250 ng of proteoglycans per well, and the wells coated with BSA served as controls in experiments. To measure lipoprotein binding, native or $sPLA_2$-V–modified lipoproteins (0.5 mg/ml) were added to proteoglycan- or BSA-coated wells. The plates were incubated for 1 h at +37 °C in a buffer containing 1% BSA, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and either 10 mM MES (pH 5.5) or 10 mM HEPES (pH 7.5). After incubation, the wells were washed three times with a buffer containing 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and either 10 mM MES (pH 5.5) or 10 mM HEPES (pH 7.5). The amounts of proteoglycan-bound lipoproteins were determined by measuring the amounts of cholesterol in each well.

NMR-spectroscopy

For the nuclear magnetic resonance (NMR) experiments, LDL (0.5 mg/ml) samples were prepared into a NMR-buffer containing D_2O, lipolyzed, pH adjusted to pH 7.5, 7.0, 6.5, 6.0, or 5.5, and NMR data acquired in the presence of fatty acid free human serum albumin (2%, w/v). The ^{1}H NMR data were obtained at 37 °C with a Varian Unity INOVA 800MHz spectrometer equipped with a $^{1}H/^{15}N/^{13}C$ triple-resonance probe head. Acquisition time of 5.0 s and a relaxation delay of 1.0 s were used with a 90 ◦ flip angle, 8.8 kHz sweep width and 384 transients. The external tube containing the reference substance,

(sodium 3-trimethylsilyl[2,2,3,3-*d*4]propionate (TSP) 40 mmol/l, $MnSO_4$ 0.6 mmol/l in 99.8% D_2O), was placed coaxially into the NMR sample tube containing 450µl of each sample. Prior to Fourier transformation, the measured free induction decays (FIDs) with 64 k data points were zero filled and multiplied by an exponential window function with a line-broadening of 0.5 Hz.

PERCH NMR software was used for all data pre-processing steps as well as for the following lineshape fitting analyses. Each spectrum was scaled according to the area of the corresponding TSP reference signal. Even though the NMR spectral shapes for the FFA-containing albumin and PLA_2-modified LDL were very similar at all pH conditions, an individual model lineshape was generated for them at each pH. These kinds of model lineshapes depict invariant spectral characteristics for molecular compounds and can be used in the analyses of component mixtures. In this particular case, the above-mentioned model lineshapes were used to analyze the ^1H NMR spectra for the mixtures of PLA_2-modified LDL and FFA-free albumin at various pH conditions. At each pH condition, comparison of the spectral analysis for the ^1H NMR spectrum of the mixture to that of the spectrum for the PLA_2-modified LDL (without albumin) lead to the information on the redistribution of FFAs and lysoPCs due to the presence of albumin.

Isolation and culture of human monocytes

Human monocytes were isolated from buffy coats by centrifugation in Ficoll-Paque gradient. Washed cells were resuspended in DMEM supplemented with penicillin and streptomycin, counted, and plated on cell culture wells. After one hour at +37°C, the non-adherent cells were removed by washing with PBS and the medium was replaced with macrophage-serum free medium (SFM), supplemented with antibiotics. Either 10 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) or 50 ng/ml of macrophage colony-stimulating factor (M-CSF) was used. Experiments were started when monocytes had been cultured for 8 days in the presence of the cytokines and had thus been differentiated into mature macrophages.

Uptake of lipoproteins by monocyte derived macrophages

Before the experiments, the culture medium was replaced with custom-made HyQ DME/HIGH Glucose medium (HyClone) having a pH of 7.5, 6.5 or 5.5, and containing antibiotics and 4 mM L-glutamine. After incubation for 1 h, ^3H-

labeled lipoproteins were added to macrophage cultures. After a 5 h incubation time, the media and cells were collected for further analysis.

Lipoprotein uptake by macrophages was determined by measuring the degradation and cell association of ^{3}H-labeled lipoproteins. Lipoprotein degradation was quantified by the measurement of trichloroacetic acid-soluble (TCA) ^{3}H-radioactivity from the incubation media. To remove any cell-surface-bound lipoprotein particles, heparin was added to the cells, and the ^{3}H-radioactivity of the heparin incubation medium was measured. The intracellular amounts of ^{3}H-labeled lipoproteins were measured by lysing the cells with NaOH, after which ^{3}H-radioactivities, giving the intracellular lipoprotein amounts, were measured.

Molecular dynamics simulations

Three lipid bilayer systems were simulated for 50 ns using the GROMACS 3.2.1 simulation package. In particular, the fully hydrolyzed 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine bilayers with varying protonation states of the liberated fatty acids mimicked three different pH values: 9.0, 7.5, and 5.5. Thus, bilayers represented changes in membrane structures going from alkaline to acidic values. After carrying MD-simulations, the simulation trajectories were analyzed to achieve computational membrane properties. Selected physical properties of these three systems were calculated as an average of the last 30 ns of the simulation trajectories. Physical properties that were calculated included: chain structure, lateral pressure profile across the membrane, area per headgroup, and electron density profiles for the whole system, water, lysoPC and fatty acid molecules, as well as for phosphorus atoms.

V. RESULTS AND DISCUSSION

1. LIPOPROTEIN LIPOLYSIS

1.1 Increased lipolysis at acidic pH

Plasma lipoproteins contain fatty acids stored in triglycerides and in cholesteryl esters, and as surface monolayer components in the side chains in phospholipids and sphingolipids as well as some amounts of free fatty acids. The hydrolysis and re-esterification of these fatty acids plays an important role in many physiological processes. The relatively new discovery of a role for PLA_2 enzymes in modification of plasma lipoproteins provides a seemingly endless array of potential biological functions that is only beginning to be appreciated.

Previously, acidic pH and dilution have been shown to enhance PLA_2-activity in human blood plasma (Costello 1990). Since intimal fluid is an ultrafiltrate of plasma, and, accordingly, the plasma-derived proteins contained in it are diluted (Smith 1990), it is conceivable that PLA_2-activity is higher in the intimal fluid. Most of the atherogenic apoB-100-containing lipoproteins are also able to enter the arterial intima and so are present in the intimal fluid. We hypothesized that the lipoprotein particles are exposed to acidic conditions and to hydrolysis by PLA_2 enzymes in the deep areas of advanced atherosclerotic lesions. We studied, *in vitro*, the effect of acidic pH on the activity of one member of the secretory PLA_2 family, $sPLA_2$-V, present in the deep acidic areas of atherosclerotic lesions (Kimura-Matsumoto 2008, Rosengren 2006b), toward lipoproteins.

We found that the activity of $sPLA_2$-V against apoB-100-containing LDL, IDL, and sVLDL particles increased at acidic pH (Figure 2, in paper III). When compared to lipolysis at pH 7.5, the degree of lipolysis of all these lipoproteins increased at pH 5.5; LDL by 1.6-fold, IDL by 1.8-fold, and of sVLDL by 1.3-fold (Figure 2, in paper III). Although $sPLA_2$ enzymes generally have a neutral pH optimum, the $sPLA_2$-V also avidly hydrolyzed lipoprotein particles at acidic pH, suggesting that higher amounts of lipolytic products may be formed by the action of $sPLA_2$-V enzyme in the local acidic areas of the arterial intima.

The activity of $sPLA_2$ enzymes is largely regulated by their ability to bind to phospholipid membranes, and $sPLA_2$-V has a unique, relatively high affinity for anionic and charge-neutral phospholipid surfaces (Lambeau 2008, Winget

2006). Low pH can induce headgroup dehydration by neutralizing the negatively charged headgroup regions, reducing the effective headgroup size. Hence, at acidic pH, the increased proton concentration could reduce the phospholipid headgroup size and enhance the charge-neutral character of PC-rich membranes, in doing so, increase the affinity of $sPLA_2$-V to its substrate. Indeed, the transition of phospholipid membrane from one phase toward another can be induced by changes in pH, ion concentrations, membrane potential, membrane proteins and hydration (Kinnunen 1991). The lateral pressure also decreases sharply at the transition between gel phase and fluid phase (Fulford 1980). Thus, the properties of phospholipid monolayer on the surface of lipoprotein particles change when moving from neutral to acidic pH, and these changes may be more favorable for the action of $sPLA_2$-V enzyme. However, reducing the headgroup size may also increase the ordering of acyl chains (in Paper II), which together with stronger lipid-lipid interactions may decrease the efflux propensity of the phospholipid substrate from the membrane into the catalytic site of several PLA_2 enzymes (Haimi 2010). In conclusion, in spite of the fact that the potential phospholipid efflux propensity may decrease, the enhanced ability of $sPLA_2$-V to bind to its more charge-neutral substrate could increase its activity at acidic pH.

To summarize, in advanced human atherosclerotic lesions, $sPLA_2$-V is found particularly in the deep areas of the lesions (Kimura-Matsumoto 2008, Rosengren 2006b), where intima is often hypoxic (Sluimer 2008), and, accordingly, intimal fluid is acidic. Our findings suggest that the lipolytic modification of apoB-100-containing lipoproteins, sVLDL, IDL, and LDL, by $sPLA_2$-V can be increased at acidic pH, and more bioactive lipolytic products, lysoPCs and FFAs, could be locally produced in the advanced atherosclerotic plaques.

1.2 LDL retains hydrolytic products at acidic pH

The most abundant plasma protein, albumin, may bind the formed lipolytic products in the intima and transfer them back to the circulatory system. The concentration of albumin present in the intimal fluid has been estimated to be about half of its plasma concentration (Smith 1990). We studied the effect of pH on the ability of albumin to remove the lipolytic products from the lipolyzed LDL particles. Using ^{1}H NMR-spectroscopy (Soininen 2007) we could directly examine the transfer of FFAs and lysoPCs to albumin in the LDL-albumin incubation mixtures. The transfer of lipolytic products was also

studied by separating albumin from LDL incubation mixtures by ultracentrifugation, and by measuring the amounts of retained lipolytic products, FFAs and lysoPCs, in LDL particles. We found that the amount of FFAs remaining in the LDL particles depended on the pH: acidity increased the amount of FFAs that remained LDL-bound (Figure 1A, in paper I and Table 1, in paper I). The amounts of lysoPCs were also determined and acidity also increased the percentage of lysoPCs that remained LDL-bound (Figure 1B, in paper I and Table 1, in paper I). Thus, at acidic pH, the ability of physiological concentration of albumin to sequester FFAs and lysoPCs from sPLA$_2$-V–modified LDL particles was decreased, and the lipolytic products were retained in LDL particles.

The observed pH-dependent transfer of FFAs to albumin may result from the property of albumin to preferably bind FFAs in their anionic form (Spector 1975, Hamilton 2002). Since the pKa of FFAs in a phospholipid membrane is approximately 7.5 (Small 1984), about half of the PLA$_2$-generated FFAs in LDL are in anionic form at pH 7.5, while all of them are uncharged at pH 5.5. Therefore, as acidity decreases the affinity of albumin for the FFAs, the transfer of the lipolytic products from lipolyzed LDL to albumin also decreases, and FFAs are retained in the lipolyzed LDL particles.

Albumin consists of 585 amino acids and has a rather small size (He 1992). It can easily pass through the intima and reach the lymphatic capillaries originating in medial layer of the arterial wall (Smith 1990). Therefore, in early atherosclerotic lesions in which the extracellular pH is physiologic, albumin has the potential to carry the PLA$_2$-generated FFAs and lysoPCs away from the arterial intima back to the circulation. In contrast, the local acidity of advanced atherosclerotic lesions may block this carrier function of albumin. Importantly, the physiological concentration of albumin in blood serum varies between individuals ranging from about 20 to about 50 mg/ml (Nelson 2000), and so the physiological concentration of albumin in the intima also must vary among individuals, constituting about half of its plasma concentration (Smith 1990). Thus, the differences in the intimal concentration of albumin between individuals may have an effect on the transfer of lipids to albumin. In our experimental system, a decrease in the albumin concentration was found to increase the accumulation of the lipolytic products in LDL particles. If this also applies to the arterial intima *in vivo*, PLA$_2$-induced modification of LDL particles in the acidic intimal fluid would have more atherogenic potential in

individuals with low serum albumin concentrations compared to individuals with high serum albumin concentrations. Hence, the function of albumin as a scavenger for FFAs and lysoPCs may partly explain the observation that low levels of serum albumin associate with an increase in CHD incidents (Nelson 2000).

In conclusion, although elevated cholesterol levels are a recognized risk factor for atherosclerosis, the quality of lipids in lipoprotein particles are also currently recognized as a risk factor for the development of atherosclerosis (Matsumoto 2007). Several phospholipid-derived bioactive compounds are generated from lipoprotein particles by phospholipases and by oxidation, including fatty acids, lysophospholipids, and phosphatidic acids. These lipids, if retained in LDL particles at acidic pH, may have various atherogenic effects upon intimal cells (Schmitz 2009, Matsumoto 2007, Macphee 2006, Wilensky 2009).

1.3 PLA_2-hydrolysis induced changes on the surface monolayer

The monolayer covering the lipoprotein particles is mainly composed of PCs, and the hydrolysis of PC by PLA_2 enzyme generates lysoPCs and FFAs. To gain information at molecular level on the effects of PLA_2-induced changes at acidic pH, we used molecular dynamics (MD) simulations to simulate three fully hydrolyzed model membranes that may reflect a physiological nanodomain on the surface of lipoprotein particles.

We found that acidity changes the properties of PLA_2-lipolyzed membrane. At acidic pH, the membrane leaflets were found to be laterally more tightly packed (Figure 2 and Figure 3, in paper II); moreover, the spontaneous curvature of the opposing monolayer leaflets became negative (Table 2, in paper II). When applied to the monolayer covering the lipoprotein particles, these properties suggest that the tendency of lipoprotein particles to fuse is pH-dependent.

Lipoprotein particles can be considered as spherical lipid droplets of a given volume. At neutral pH, the FFAs on the surface of droplets are partially negatively charged and more hydrophilic. Therefore, they tend to lower the surface tension of an oil-water interface. At low pH values, however, the FFAs are uncharged and less hydrophilic than at neutral pH, and, in such a droplet, acidity could lead to increased surface tension. The increased surface tension can return to the original value if lipid molecules absorb on the surface, or if

the particle size decreases. However, these two processes are likely to be too slow in order to decrease the surface tension of a droplet, when compared to the rapid fusion of membranes induced by increased surface tension (Lyklema J. 2000). Taken together, our results suggest that local extracellular acidity and lipoprotein lipolysis by PLA_2 may promote lipoprotein fusion by inducing negative spontaneous curvature to the lipolyzed monolayer covering lipoprotein particles, and also by increasing the surface tension of the lipoprotein particles.

2. LIPOPROTEIN RETENTION

2.1 Binding of lipoproteins to proteoglycans

In the intima, lipoprotein particles can become modified and bound to the arterial proteoglycans. The subendothelial retention of lipoprotein particles is the initiating event in atherosclerosis, and, once retained, the lipoprotein particles provoke a cascade of responses that lead to atherosclerosis in a previously non-lesional artery (Tabas 2007).

To determine if acidic pH and the lipolysis of lipoprotein particles with $sPLA_2$-V enzyme can have an effect on the binding of lipoproteins to proteoglycans, we used microtiter wells coated with proteoglycans isolated from the intima-media of human aortas. We found that acidity increased the amounts of proteoglycan-bound native LDL, IDL, and sVLDL (Figure 3, in paper III). Thus, at pH 5.5, there was 270-fold increase in the binding of native LDL, a 60-fold increase in the binding of native IDL, and a 140-fold increase in the binding of native sVLDL to proteoglycans, as compared to binding results at pH 7.5. Additionally, we found that at acidic pH 5.5, lipolysis of lipoprotein particles by $sPLA_2$-V further increased the amounts of proteoglycan-bound lipoproteins (Figure 3, in paper III). Thus, at pH 5.5, $sPLA_2$-V–LDL bound 1.9-fold, $sPLA_2$-V–IDL bound 1.7-fold, and $sPLA_2$-V–sVLDL bound 2.0-fold more than native lipoprotein particles at the same pH.

In conclusion, we found that acidic pH induced a dramatic increase in the binding of native and $sPLA_2$-V-lipolyzed apoB-100-containing plasma lipoproteins, sVLDL, IDL, and LDL particles, to human aortic proteoglycans. The acidity-induced increase in the binding of all three classes of native lipoprotein particles to human aortic proteoglycans can be mediated via the ionic interactions between the negatively charged glycosaminoglycan chains of the proteoglycans and the positively charged amino acids lysine and arginine of

the apoB-100 of the lipoprotein particles, which has been previously observed in the case of LDL (Sneck 2005). In contrast, PLA_2-lipolysis increases the binding of LDL to proteoglycans due to an alteration in the conformation of apoB-100, which exposes a new proteoglycan-binding site (Flood 2004). This binding site may also be responsible for the observed increased binding of the PLA_2-lipolyzed IDL and sVLDL particles to proteoglycans. Moreover, at low pH 5.5, the FFAs of lipoproteins are protonated (Kanicky 2002), which increases the charge difference between the lipoprotein particles and the negatively charged proteoglycans. This increase in electrostatic interactions may lead to stronger interactions between the apoB-100-containing plasma lipoprotein particles and the proteoglycans at acidic pH.

2.2 Affinity of lipoproteins for proteoglycans

Using proteoglycan-affinity column chromatography, we eluted the proteoglycan column bound lipoproteins with increasing salt concentration. We found that, both at neutral and at acidic pH, native LDL (Figure 4C, in paper III) eluted from the column as a single peak, whereas native IDL (Figure 4B, in paper III) and sVLDL (Figure 4A, in paper III) eluted in two or three peaks, revealing heterogeneity among the IDL and sVLDL particle classes. We also found that the binding affinity of the LDL, IDL, and sVLDL particles was significantly higher at acidic pH than at neutral pH. Thus, the major fraction of all studied apoB-100-containing lipoproteins required a supraphysiological NaCl concentration (400 mM) for detachment from the human aortic proteoglycans. Regarding lipolysis, $sPLA_2$-V had the largest effect on the affinity of LDL for proteoglycans (Figure 4C, in paper III), while its effect on the affinity of VLDL (Figure 4A, in paper III) and IDL (Figure 4B, in paper III) for proteoglycans was smaller.

In all three lipoprotein classes, sVLDL, IDL, and LDL, lower amounts of particles failed to bind to proteoglycans at acidic pH than at neutral pH (Figure 4A-C, in paper III). This indicated that a fraction of the lipoproteins not binding at pH 7.5 did bind at pH 5.5, which further implicated their higher binding affinity for proteoglycans at acidic pH. Taken together, we found that acidity had dramatic effect on the binding affinity of apoB-100 containing lipoproteins for proteoglycans, which suggests that acidity may increase the proteoglycan binding of the three classes of lipoprotein particles in atherosclerotic lesions.

2.3 Effect of small apolipoproteins on sVLDL binding to proteoglycans

The binding of LDL, IDL, and sVLDL to proteoglycans is mainly mediated by the interaction between apoB-100 and proteoglycans. In addition, IDL and VLDL particles also contain additional small apolipoproteins, such as apoC-III and apoE, which are potentially capable of influencing the proteoglycan-lipoprotein interaction, since the apoE moiety of lipoproteins has been shown to bind to proteoglycans (Camejo 1988).

We used proteoglycan-affinity column chromatography to further study the relationship between the apoC-III and apoE contents of the sVLDL particles and their relative affinities for proteoglycans. As discussed above, sVLDL eluted from the proteoglycan column in three populations (Figure 4A, in paper III). From these three populations, we analyzed the apoC-III and apoE contents and found that the sVLDL particles with low affinity had high contents of these two apolipoproteins (Figure 5A-B, in paper III), and the highest apoC-III and apoE contents were found in sVLDL particles that had the lowest affinity toward proteoglycans at acidic pH. Notably, these particles were not able to bind to proteoglycans even at acidic pH, which favors the electrostatic interactions between lipoproteins and proteoglycans. We also used an anti-apoE affinity chromatography to separate apoE-enriched particles and found that the separated particles showed only minor binding to proteoglycans in our proteoglycan coated microtiter well assay (Figure 6, in paper III).

To summarize, we found that the particles with the lowest affinity for human aortic proteoglycans had the highest amounts of apoE and apoC-III. In contrast, it has previously been shown that the binding of proteoglycans to VLDL, derived from different healthy donors, correlates positively with the apoC-III content of this lipoprotein class (Olin-Lewis 2002). This correlation is surprising since apoC-III does not itself bind to proteoglycans, and addition of purified apoC-III to apoB-containing lipoproteins *in vitro* has been shown to inhibit the lipoproteins binding to proteoglycans (Olin-Lewis 2002). It has been suggested that the previously observed positive correlation between apoC-III content and the proteoglycan binding to lipoproteins relies on apoC-III-dependent conformational changes in apoB-100 (Olin-Lewis 2002, Hiukka 2009). Our findings, showing that lipoproteins having the highest apoC-III content actually have the lowest affinity for proteoglycans, favor the view that, if there is a high apoC-III content in the total particle population of sVLDL lipoprotein class, it may reflect some other intrinsic property, that affects both

their apoC-III content as well as their binding to proteoglycans. Such an intrinsic property can be particle size, as apoC-III and apoE are more enriched on large VLDL particles (Marcel 1988, Shin 2010), and large VLDL particles have been shown to have a lower affinity for proteoglycans than small VLDL particles (Anber 1997). Additionally, higher apoE and apoC-III contents at the lipoprotein particle surface may reflect the surface pressure of these lipoprotein particles, since the association of small apolipoproteins to the particle surface is sensitive to the surface pressure of the phospholipid monolayer covering the particles (McNamara 1996). Altered surface pressure can result from changes in lipid composition on the particle surface, due to extensive modifications or abnormal endogenous lipid constituents. The addition of some small apolipoproteins to the surface may return the surface tension to the normal value, without changing the properties of lipids.

In conclusion, we observed that LDL, IDL, and sVLDL particles consist of heterogeneous populations of lipoprotein particles, with lower and higher binding affinities for human aortic proteoglycans. We also observed that the sVLDL class of lipoprotein particles is heterogeneous in apoE and apoC-III contents, suggesting that the apoE and apoC-III contents in a particle reflect an intrinsic property of the particle that also affects binding affinity for human aortic proteoglycans.

3. INTRACELLULAR LIPID ACCUMULATION

3.1 Lipoprotein uptake by macrophages

At all stages of atherosclerosis, arterial macrophages take up lipoproteins by an unregulated mechanism leading to intracellular lipid droplet accumulations. Under a microscope, these cells have a foamy appearance and are called “foam cells.”

The present investigations revealed that lipoprotein lipolysis by $sPLA_2$-V enzyme leads to increased particle uptake by macrophages at acidic pH and to foam cell formation (Figure 4, in paper I and Figure 7, in paper III). First, with macrophages differentiated with GM-CSF, we found that $sPLA_2$-V–modified LDL is avidly taken up by macrophages at acidic pH (Figure 4, in paper I). Thus, the elevated amounts of cell-associated and degraded $sPLA_2$-V-modified LDL particles were 1.5-fold higher at pH 6.5, and 3.5-fold higher at pH 5.5, when compared to levels at pH 7.5. Secondly, with the macrophage phenotype predominating in the arterial intima, i.e., macrophages differentiated with M-

CSF, we found that acidity did not increase the intracellular accumulation of native LDL, IDL, and sVLDL particles (Figure 7A-C, in paper III). In fact, acidity decreased the uptake of native sVLDL (Figure 7A, in paper III). This observed decrease in the uptake of native sVLDL by macrophages at acidic pH may depend on the properties of VLDL receptor. Thus, at neutral pH, the apoE-containing lipoproteins can bind to the VLDL receptor (Takahashi 2004), but the lipoprotein ligands are released from the LDLR family at low pH (Fisher 2006, Rudenko 2002), which may partly explain the observed decreased uptake of sVLDL particles.

We also found that lipolysis with sPLA$_2$-V of all three lipoprotein classes (LDL, IDL, and sVLDL particles) resulted in their increased uptake and increased intracellular lipid accumulations in macrophages (Figure 7A-C, in paper III). Thus, in our experimental system, macrophages were found to avidly take up sPLA$_2$-V-modified LDL, IDL, and sVLDL lipoprotein particles both at neutral and at acidic pH (Figure 7A-C, in paper III). The possible mechanism for the increased lipid uptake at acidic pH is not likely to involve the LDLR-related family of receptor proteins or LRP-1, because of their nature to bind ligands at neutral pH (Fisher 2006, Rudenko 2002, Moestrup 1990). However, since the binding strength of sPLA$_2$-V-modified LDL to proteoglycans is increased at acidic pH (Figure 3B, in paper I and Figure 3, in paper III), it is possible that the observed increased lipoprotein uptake depends on increased binding to cell-surface proteoglycans. Indeed, recently, at neutral pH, the uptake of sPLA$_2$-V-modified LDL was shown to depend on cell-surface proteoglycans, particularly on syndecans (Boyanovsky 2005, Boyanovsky 2009a). We confirmed this observation by first treating macrophages with a combination of chondroitinase and heparinase and found that the binding of LDL to cell-surface was after this treatment decreased at acidic pH.

In conclusion, acidity failed to increase the uptake of native lipoproteins by human monocyte-derived macrophages. In contrast, premodification of all three lipoprotein classes with the sPLA$_2$-V enzyme was able to induce such modifications at the lipoprotein particle surface that they were internalized by macrophages, both at neutral and at acidic pH values. The combined effects of both acidity and lipoprotein lipolysis by sPLA$_2$-V enzyme may thus lead to intracellular lipoprotein accumulations.

4. FUTURE PERSPECTIVES

To date, the most effective pharmacotherapy against atherosclerosis are statins, which are based on the principle that decreasing the amount of circulating LDL particles decreases the probability that they will enter, and be retained in, the arterial subendothelium. Ongoing improvements in this area include more aggressive lowering of LDL and other atherogenic lipoproteins in the plasma, and initiation of the therapy in high-risk individuals at an earlier age. However, statins alone, even in high doses, are not sufficient to fully prevent the progression of atherosclerosis in many susceptible individuals. Indeed, cardiovascular events continue to occur in two thirds of all patients on statin treatment, as has been shown by several large controlled clinical trials, including the Scandinavian Simvastatin Survival Study, the Cholesterol and Recurrent Events trial, the Air Force/Texas Coronary Atherosclerosis Prevention studies, and the Long-term Intervention with Pravastatin in Ischemic Disease study (Libby 2005). Thus, there a real need exists for a deeper understanding of the pathophysiological mechanisms by which lipoprotein particles in the intima accelerate the progression of atherosclerosis.

The key initiating process in atherogenesis is the subendothelial retention of apoB-100-containing lipoproteins. New approaches, as future therapeutics, may include attempts to block the interaction of apoB-100-containing lipoproteins with the specific subendothelial matrix molecules that promote lipoprotein retention. The matrix-retained lipoprotein particles are prone to several types of modifications: oxidation, proteolysis and lipolysis. The blocking of molecules within the arterial wall that modify lipoprotein particles are potential therapeutic targets as well. Local biological responses to these retained and modified lipoproteins, including chronic inflammation and fatty streak formation, also promote the subsequent lesion development.

There is ample evidence that enzymatic particle lipolysis, which can also be coupled to particle oxidation, is a particularly atherogenic process. Thus, significant amounts of lipids are stored as triglycerides in macrophages (Mattsson 1993), and hypoxia increases the amounts of cytosolic lipid droplets with increasing amounts of triglycerides (Boström 2006), suggesting that, in addition to cholesterol, fatty acids are taken up and stored by macrophages as triglycerides. However, because of the complexity of cellular lipid metabolism and its regulation, it has been difficult to establish a causal link between specific lipid mediators within the vessel wall and clinical events.

Recent discoveries suggest that lipoprotein particles can be extensively modified by $sPLA_2$ and Lp-PLA_2 enzymes, and blocking the action of these enzymes may represent a new approach to the treatment of atherosclerosis. Thus, it is not only the quantity of cholesterol circulating and able to enter the intima, but also the quality of various other lipids in lipoprotein particles, that can be strikingly atherogenic. The observed potential links between free fatty acids produced by $sPLA_2$-V enzyme and the extracellular and intracellular accumulations of lipoprotein particles, which may lead to the development of foam cells in atherosclerosis, offer future targets for PLA_2 inhibitors. Inhibitors that are specific for atherogenic PLA_2 enzymes are the most promising, and the development of these inhibitors has become the focus of pharmacological research in this last decennium (Garcia-Garcia 2009, Oslund 2008). Currently, there are several ongoing Phase III clinical trials with the PLA_2 inhibitors (Suckling 2009). These inhibitors are specific for Lp-PLA_2, or for several forms of $sPLA_2$s. There is an ongoing STABILITY trial with Darapladib (GlaxoSmithKline), which is an inhibitor of Lp-PLA_2 (Riley 2009, Bui 2010). Varespladib (Anthera), inhibits the $sPLA_2$-IIA, $sPLA_2$-V and $sPLA_2$-X, and there is an ongoing FRANCIS-ACS trial on patients with acute coronary syndrome (Karakas 2009). Thus, inhibitors of the enzymes that play critical roles in LDL modification are potential novel antiatherogenic drugs that could be used synergistically with statins. They may also offer new treatment options to those patients who fail to gain the full benefit from statin treatment.

VI. SUMMARY AND CONCLUSIONS

This thesis aimed to elucidate some of the atherogenic effects of acidic pH on the apoB-100-containing lipoprotein accumulation in the arterial intima, with a specific focus on the role of $sPLA_2$-V enzyme. Based on our findings and the above discussion, the following conclusions can be presented. Figure 5 recapitulates the main findings in an illustrative way.

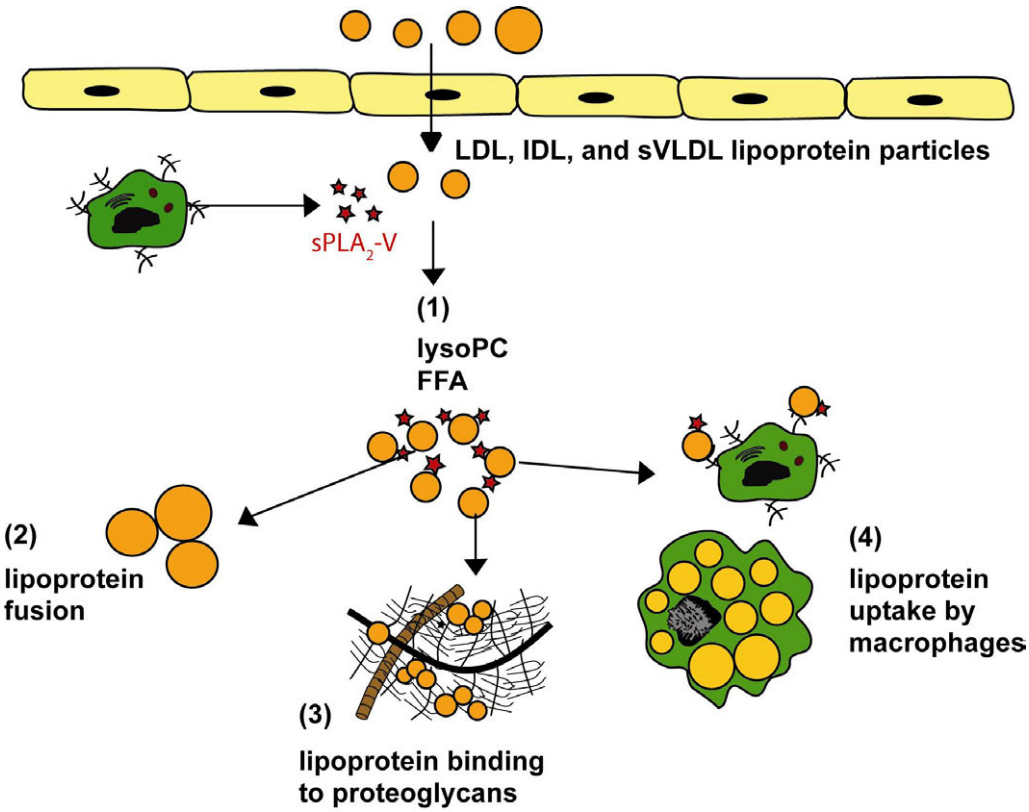

Figure 5. Summary of the main findings of the thesis. (1) Acidity increases the apoB-100-containing lipoprotein particle lipolysis by $sPLA_2$-V enzyme, and the lipolytic products, lysoPCs and FFAs, are retained in LDL particles. (2) At acidic pH, the tendency of the apoB-100-containing lipoprotein particles to fuse into extracellular lipid droplets increases after PLA_2 hydrolysis. (3) Lipolysis by $sPLA_2$-V enzyme and acidity increase the apoB-100-containing lipoprotein particle binding to extracellular matrix proteoglycans. (4) At acidic pH, the $sPLA_2$-V-modified apoB-100-containing lipoprotein particles are taken up by monocyte-derived macrophages, thus promoting the formation of foam cells.

Current evidence suggests that the development of atherosclerosis culminates in a gradual increase in lipid deposition and ensuing chronic inflammation in the subendothelium (Williams 1995, Libby 2002). The subendothelially deposited atherogenic lipoproteins provide substrates for multiple modifying agents. The modified lipoproteins are subsequently taken up by locally

recruited macrophages, which are then converted into foam cells. Foam cells, in turn, are activated to produce various cytokines that promote and maintain the local inflammation (Boyanovsky 2010). The $sPLA_2$ family of enzymes has been implicated in pro-atherosclerotic processes because of their hydrolyzing activities in the arterial intima (Rosenson 2009a), and because diet-induced or gene-induced hyperlipidemia in mice enhances the expression of $sPLA_2$-V in aorta (Rosengren 2006b). Our results suggest that $sPLA_2$-V enzyme can be considered a particularly atherogenic enzyme in the acidic microdomains of advanced atherosclerotic plaques. At acidic pH, $sPLA_2$-V is able to hydrolyze plasma lipoprotein particles (papers I and III), thereby leading to increased amounts of retained bioactive hydrolysis products, FFAs and lysoPCs, in LDL particles (paper I). After lipolysis, the lipoprotein particles have a higher binding strength toward the arterial proteoglycans (papers I and III). The PLA_2-lipolyzed lipoprotein particles are also more prone to fuse at acidic pH, because of the altered, fusion-promoting properties of the surface monolayer of the lipolyzed particles (paper II). At acidic pH, the retained and hydrolyzed lipoprotein particles are taken up by monocyte-derived arterial macrophages, which tends to lead to the formation of foam cells (papers I and III).

In conclusion, the extracellular acidic pH of advanced atherosclerotic lesions may provoke increased lipoprotein lipolysis. Furthermore, the lipolyzed particles strongly bind to arterial proteoglycans and are taken up by arterial macrophages. However, even though acidity strongly increased the binding of native lipoproteins to human aortic proteoglycans, it failed to increase the uptake of native lipoproteins by human monocyte-derived macrophages. In contrast, the premodification of all three lipoprotein classes with $sPLA_2$-V enzyme was able to render such modifications at the lipoprotein particle surface that they were internalized by macrophages, both at neutral and acidic pH values. Hence, the combined effects of acidity and lipoprotein lipolysis by $sPLA_2$-V enzyme may, together, lead to both intracellular and extracellular lipoprotein accumulations.

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